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Ole Maaløe

ON THE RELATION BETWEEN

ALEXIN &
OPSONIN

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COPENHAGEN 1946

Thesis

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ALEXIN &
OPSONIN



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EINAR MUNKSGAARD

COPENHAGEN 1946

*Denne Afhandling
er af det lægevidenskabelige Fakultet
antaget til offentlig at forsvares
for den medicinske
Doktorgrad.*

København, den 4. April 1946.

H. C. A. LASSEN,
h. a. dec.

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KØBENHAVN

PREFACE

The present work has been carried out in the State Serum Institute in Copenhagen. First of all I wish to thank J. Ørskov, M.D., chief of the Institute, for the very favourable working conditions afforded me and for the kind interest which, on many occasions, he has shown my work.

To the progress of the work, however, the great readiness with which Dr. P. Krag, chief of the sero-diagnostic department, has always discussed theoretical and technical problems with me has proved to be the best and most direct help I could possibly receive. I wish here to thank him heartily.

It is a pleasure, too, for me to thank Professor K. A. Jensen, M.D., chief of the Pathological Institute of the University of Copenhagen, for the valuable suggestions I have received in the course of several discussions.—Furthermore Professor K. A. Jensen and Martin Kristensen, M.D., chief of the bacteriological department of the Serum Institute, have given me much needed advice as to the form of publication for which I am indebted to both of them.

G. Rasch, Ph.D., and Dr. G. Wegener have helped me in arranging and translating the statistical part of the book and I am glad here to convey my thanks.

Finally, I want to mention the help I have received from several of the assistants of the Serum Institute, especially the two donors who have been ready at any time to supply me with fresh blood. To all who have helped me I should like to offer my best thanks for their kind assistance.

Copenhagen, May 1946.

OLE MAALØE

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Translated from Danish
by
 AXEL ANDERSEN
Copenhagen

PART ONE

CHAPTER I

INTRODUCTION

The object of the present work is to communicate some results of experiments performed with a new technique for determinations of bactericide and phagocytosis. First and foremost comparisons have been made between the course of the bactericidal and phagocytosis processes under varying external conditions, because by this means the writer wants to contribute towards the solution of the question whether ALEXIN and OPSONIN—i.e. the thermolabile antibacterial normal substances of the serum—may be considered identical or not.

The reason why this question is taken up is, to some extent, that the technique worked out by the writer is especially advantageous to such comparative work: In contradistinction to previous methods of examination, necessitating determinations of the degrees of bactericide and phagocytosis by means of culture and microscopy respectively, the new technique makes it possible for the two processes to be examined in a common milieu and with a common criterion. This has been achieved by utilizing the bacterial density at the beginning and at the end of the experiment as a means to express *both* the degree of bactericide and that of phagocytosis. In order to let the phagocytosis process be reflected in this manner a slight centrifugation is interposed towards the end of the experiment, the blood corpuscles + the phagocytized bacteria thus being thrown down, whereas the free bacteria are only comparatively slightly influenced.

Another important point is that the new technique renders work with very weak bacterial suspensions possible. This means that the examinations can be performed irrespective of what the cultures employed may contain of agressin-like or leukocyte-

injuring substances; owing to the weak dilution in which such substances occur in the reaction milieu they will be of no importance to the results of the experiments. In experiments with addition of immune serum, too, it is advantageous to work with so slight a bacterial density; as will be shown in the following experiments with a slight bacterial density can be carried through without any risk of agglutination.

The discrimination between the concepts of alexin and complement has been somewhat varying; in the present publication the following discrimination is made: Referring to the *bactericidal process* the term ALEXIN is used about the active serum substance, as originally stated by Buchner when he described the bactericidal properties of cell-free serum. Referring to the *hemolytic process* the term COMPLEMENT is used about the active substance in conformity with Anglo-American usage. A number of the investigators who have been engaged most in alexin and complement examinations have, however, arrived at the result that the bactericidal and hemolytic substances are identical (e.g. Gordon 1928, Eagle 1929, and Muir 1931). In the present publication no new facts in support of this view will be set forth, but based on the results of previous investigations the words alexin and complement will be considered two terms for the same substance in the following, although used each in its own connection. As to the word OPSONIN no further definition is required; since it was introduced by Wright it has only been used to designate the thermolabile phagocytosis-stimulating normal substance of the serum.

It is of importance to emphasize that investigating the anti-bacterial serum functions we are at present still working with substances—or rather complexes of substances—that are chiefly known and described through their effects. It has hitherto only been managed roughly to state chemical characteristics of the active substances, and a comparison like the one it is desired to make here between alexin and opsonin must therefore, as already suggested, first and foremost be a comparison between the course of the bactericidal and the phagocytosis-stimulating processes.

In the following a short survey of the alexin and opsonin research will be given*. As far as more special works are concerned the references to literature attached to the descriptions of the experiments in the subsequent chapters must be consulted.

The antibacterial effect of the blood is first mentioned by Nuttall (1888), whereas as already mentioned Buchner (1889) showed that the bactericidal property is associated with the cell-free plasma or serum; Buchner gave the effective substance the name of alexin. Among the observations that became of importance to the subsequent splitting up of the alexin in components it will be mentioned that Buchner (1889) and Buchner and collaborators (1890) showed that dilution of serum with distilled water or dialysis against water discontinues the alexin effect.

Ferrata (1907) communicated that he was able to separate complement into a globulin and an albumin fraction; the latter was found to remain in solution when the globulins were precipitated. Both of these protein components are *thermolabile*, being destroyed in about 10 min. at 56°. Soon after the publication of Ferrata's communication Brand (1907) showed that the globulin fraction—the so-called *midstuk*—can be bound by sensitized blood corpuscles, which after washing can be hemolyzed by the albumin fraction—the *endstuk*—alone. The method most frequently used to separate midstuk and endstuk is dilution of the serum with distilled water and subsequent passage of CO₂ through the fluid; it should, however, not be taken for granted that, by means of such precipitation of the globulins, two well-defined components, each with its own function in the hemolysis process, are actually separated.

When the investigations referred to were published in 1907 the opsonin doctrine was but few years old. Though it had been known for long that serum is phagocytosis-promoting, it was not until 1904 that Wright showed decisively that the serum effect is due to a thermolabile substance—opsonin—which through influence on the bacteria renders the latter more phagocytal. Wright's discovery soon led to the abandonment of Metchnikoff's

* As a more extensive introduction to the alexin research a collective work by Osborn (1937), "Complement or Alexin" can be recommended.

theory that the serum effect should be due to leukocyte stimulation.

From the onset alexin and opsonin were considered closely related or identical (see for instance Levaditi & Inmann, 1907). Special attention was paid to the facts that besides being thermolabile the two substances can be removed from serum by means of absorption with bacteria and that the effect of both substances can be increased by specific immune serum. In support of the resemblance between alexin and opsonin Hata (1908) was able to show that opsonin, too, requires the concurrence of both a globulin and an albumin component.

In the present work no examinations of the thermolabile protein fractions were made separately. The part of the investigations dealing with these components was performed by means of so-called anticomplementary (self-inhibitory) serum; sera of this nature exercise a highly inhibitory effect on the alexin and opsonin activities, and in the writer's opinion the inhibition is due to one or both of the thermolabile components being adsorbed to abnormal substances in the anticomplementary serum (see Chapter VIII).

Continued studies—especially of the hemolysis process—have disclosed still two components and it has been shown that the two of them are indispensable to both the hemolysis and the bactericidal processes:

The third component, which can be separated independently on the basis of works by Omorokow (1911) and Sachs & Omorokow (1911), is characterized by being comparatively *thermostable* and liable to be destroyed by cobra venom or yeast cells. Already in 1907 Neufeld & Huhne showed that both the alexin and opsonin effects were discontinued when serum was treated with yeast, and from this fact it was later concluded that the third component is contained both in alexin and opsonin. On precipitation of the midstuk and endstuk the third component is also thrown down and it must thus be supposed to be of protein character.

The fourth component has been described by Gordon, Whitehead & Wormall (1926) and its most important characteristic is that it is destroyed when treated with ammonia. The fourth

component is *thermostable* to a somewhat greater extent than the third (Ecker, Pillemer & Kuehn, 1940). That the third and fourth components are not identical was shown by Gordon and collaborators in reactivation experiments proving that a serum that does not contain the third component can be activated by a serum treated with heating and NH_3 and that a serum not containing the fourth component can be activated by one treated with cobra venom and then heated. Deissler (1932) has demonstrated another important difference between the two thermostable components: He showed that the fourth component must be present when binding between the midstuk and the sensitized blood corpuscles takes place and that the third component will only begin to act when this binding has taken place.

Through different experiments Gordon and collaborators (1926, 1929, and 1935) tried to show that the fourth component is not required in the opsonin process. These investigations constitute an important feature of recent alexin and opsonin research and are quoted by several authors (Muir 1931, Osborn 1937, and Jersild 1942) as a proof that, despite the resemblance, there is a qualitative difference between alexin and opsonin. Gordon's experiments will be referred to in detail in connection with the reference to the writer's own experiments with serum treated with NH_3 (see Chapter VII).

As to the fourth component it will lastly be mentioned that Toda & Misuse (1933) and Tokano (1936) have shown that by shaking serum with ether or chloroform a factor that presumably is identical with Gordon's fourth component can be removed wholly or in part.

Besides the investigations into the said 4 components numerous communications about the effect of different electrolytes on the bactericidal and hemolytic processes have been published in the course of time (most authors have been working with alexin and complement investigations, which are technically easier than phagocytosis investigations). Nissen (1889) and later on Löwit & Schwarz (1903) showed that sulfates inhibit the bactericidal process; Pettersson (1902), Falloise (1903) and several others examined the activity in citrate and oxalate plasma, and Sachs & Teruuchi (1907) and Tokunaga (1928) communicated investiga-

tions into the effect of numerous different salts on the hemolytic process. More recently some authors have examined the importance of Ca to the complement activity (Gordon, Whitehead & Wormall 1926, Wadsworth, Maltaner & Maltaner 1936 and several others). A number of the publications referred to will be dealt with in detail in connection with the writer's own investigations into the effects of citrate, oxalate, tartrate, and sulfate on the alexin and opsonin processes (see Chapter IX).

Besides the components of which alexin, complement, and opsonin are made up—and among which Ca perhaps should be included, as will be established in the following—it is necessary to mention still another factor; the latter does not actually form part of any of the active serum substances but constitutes *an indispensable intermediate* between these and the bacteria or blood corpuscles that have to be influenced. This intermediate is best known from the hemolytic process in which it is still referred to with the old term *amboceptor* and generally added in the form of specific immune serum produced by means of immunization of rabbits with ovine erythrocytes. The addition of specific amboceptor is necessary when guinea-pig serum is used as complement source, as guinea-pig serum does not contain “natural” antibodies against ovine erythrocytes; other sera, e.g. certain human sera, contain such “natural” antibodies and, if they are present in sufficient quantities, the serum will be capable of hemolyzing ovine blood without preceding “sensitization” of the erythrocytes with amboceptor.

As far as the antibacterial processes are concerned it has been proved through numerous works that both the bactericidal and the phagocytosis process require the concurrence of a thermostable antibody-like factor (see Chapter V).

It is the experience of most authors that this factor that is of spontaneous occurrence—the “normal antibody”—is rather unspecific and it is known that it can be supplemented or replaced by a specific immune body which, like the amboceptor, is procured in the form of serum of an animal or an individual that has been actively immunized.

To demonstrate the presence of the “normal antibodies” the fact has been utilized that the binding between bacteria and anti-

body may take place at 0° . Absorption of the normal antibody may thus be effected at a temperature where alexin and opsonin are inactive and therefore are not involved in the process. If absorption is made at temperatures between 20° and 40° alexin and opsonin will begin to act, and if the adsorption is made with sufficiently great amounts of bacteria it will be possible, as referred to, to empty the serum completely of alexin and opsonin.

The writer's examinations into the functions of the normal and the specific antibodies in the bactericidal and the phagocytosis processes will be found in Part II (Chapters IV and V). Here, too, is explained what is understood by a "normal" serum in the present work and by which criteria it is made sure that sera which are considered normal do not contain immune substance (p. 94).

In the Table on next page it is undertaken on the basis of the literature to give a survey of all the factors necessary to the alexin and opsonin processes and of some of their most important properties.

It will appear from the survey of literature that comparisons between alexin and opsonin have hitherto been made in a very disconnected manner, most frequently in connection with new discoveries in the domain of complement research. Summing up the results hitherto obtained the following conclusion is arrived at: Alexin and opsonin resemble each other first and foremost in being both of them thermolabile antibacterial serum substances of normal occurrence. Secondly, both substances are of a complex constitution containing certain components in common and, lastly, both the alexin and the opsonin processes require the concurrence of an antibody-like factor (normal or specific antibody). According to the literature only Gordon's and his collaborators' experiments speak against the idea of complete identity between alexin and opsonin, as their experiments seem to show that the fourth component is included in alexin (and complement) but not in opsonin.

The experiments hitherto performed to compare alexin and opsonin have generally been on a broad basis so that one or more species of serum have been examined each with a number

	<i>Midstuk</i>, precipitated with the globulins on dilution and treatment with CO₂.	
<i>Thermolabile</i>, destroyed in about 10 min. at 56°.	<i>Endstuk</i>, precipitated with the albumins; the distinction between the glob. and alb. fractions possibly artificial.	Together they constitute the "active substance" alexin or complement. The first three components are moreover included in the opsonin, which according to Gordon does not require the concurrence of the fourth component.
	<i>Third component</i>, destroyed by cobra venom or yeast cells. Is supposed to be the last acting component.	
<i>Thermostable</i>, can stand 56° for at least 10 min. but otherwise thermostable to a varying degree; third component the least, the antibody the most stable.	<i>Fourth component</i>, destroyed by NH₃, extracted or destroyed by ether or chloroform. Supposed to be necessary to the first part of the hemolytic process.	Alexin, complement, and opsonin can be absorbed (or consumed?) by sensitized bacteria or blood corpuscles at temperatures between about 20° and 40°.
	<i>Antibody</i> (sensitizer); in the alexin and opsonin processes in the form of "normal" or specific antibody; in the hemolytic process as a rule in the form of specific amboceptor.	Necessary intermediate between bacteria or blood corpuscles and the "active serum substance". Can be absorbed by non-sensitized bacteria or blood corpuscles at 0°.

Note: The so-called thermostable bactericidal substances (β -lysin, leucocidin and the like) fall completely outside this table.

of different bacteria. On the other hand the experiments have been performed by many different investigators and the technique employed has changed. When the question about the resemblance between alexin and opsonin is now taken up again, the writer therefore believed that it would be most expedient, once for all and with a fixed technique, to examine several different conditions thoroughly. This experimental scheme could not be carried through without abandoning the experiments on a broad basis; the requisite limitation of the extent of the experiments was achieved by using only one bacterial type and a few human sera for the experiments.

The bacterial type used is the *Salmonella* Breslau (*S. typhi* murium), which proved to be especially suitable for experiments with the new technique. Different variants of the *Salmonella* Breslau were used; the latter, which are termed Breslau 1, 50, and 206, were kindly left to me by Professor K. A. Jensen. Breslau 1 is the original strain and possesses the characteristic high virulence against mice; Breslau 50 and 206 resulted from 50 and 206 passages respectively in bouillon at increasing temperature of cultivation; Breslau 50 is slightly, Breslau 206 practically avirulent against mice. It must be emphasized that despite the great difference between the virulence of the strains no serologic difference between the three forms employed can be demonstrated. This fact was demonstrated by Kauffmann soon after the strains had been isolated, and the writer repeated the identification under Kauffmann's guidance when the present work was commenced.

As will appear from the experiments the three Breslau strains display a different sensitivity to alexin and opsonin. The virulent Breslau 1 is slightly sensitive, Breslau 50 more, and Breslau 206 the most sensitive. This different sensitivity has been of great practical importance to the planning of the experiments, many of which could not have been carried through if for instance only the slightly alexin-sensitive Breslau 1 had been at the writer's disposal.

The Breslau strains employed have another advantageous property; they are not appreciably influenced by the so-called ther-

mostable bactericidal substances in serum—i.e. β -lysins, leukocidins and the like. This property made it easy to confine the investigations to comprise the thermolabile antibacterial serum functions. The thermostable bactericidal substances, which have recently been thoroughly examined in this country by Hjorth (1938) and Ostenfeld (1944), will not be dealt with in the present publication.

Although the writer thus has had to refrain from carrying through the experiments with a representative selection of bacteria, it is still believed that a certain universal validity can be attributed to the results arrived at. The reason hereof is that almost all the facts dealt with, and examined here more thoroughly than previously, are of universal validity and well-known from the works of former researchers on a broader basis, (cf. Chapters VII to IX).

In conclusion it will be mentioned that in the survey of literature the writer has purposely omitted publications that deal with investigations into the nature of the alexin process and the effect of alexin *in vivo*. Especially the latter problem is of great importance to the understanding of the mechanism of the normal antibacterial defence, and the writer hopes later on to be able to communicate the results of investigations that are being made with a view to this question.

CHAPTER II

EXPERIMENTAL TECHNIQUE AND EVALUATION OF EXPERIMENTS

All the experiments that will be described in the following can be classified in 3 groups:

1) *Bactericidal experiments* in which the test tubes contain fresh serum, bacteria, and saline solution. The active constituent of the system is *alexin*, as the effect of the opsonin present cannot be observed because there are no blood corpuscles in the tubes.

2) *Serum phagocytosis experiments* in which the test tubes contain fresh serum, bacteria, and saline solution + an addition of blood corpuscles (red and white). The characteristic feature of these experiments is that *opsonin* and *alexin* have been added to the system and that the effects of both substances are reflected.

3) *Spontaneous phagocytosis experiments*, for which washed blood corpuscles are used which are re-suspended either in saline solution (the experiment is termed *saline-phagocytosis*) or in inactivated serum + saline solution. The characteristic feature of the spontaneous phagocytosis experiments is that the system *contains neither opsonin nor alexin*.

All 3 types of experiment can be varied by adding, instead of saline solution, reagents whose promoting or inhibitory action on the bactericidal or phagocytosis processes it is desired to examine. For practical reasons all test tubes have one thing in common: The volume of the fluid phase is constantly kept = 1.0 cc. The fact that the volume of the fluid phase and not that of the total experimental milieu is kept at a constant level is of importance to the direct comparison between tubes with and tubes without blood corpuscles. As a rule only soluble substances will influence the bactericidal or phagocytosis process, and as most efficacious substances are protein-like products of a high

molecular weight which do not permeate the membrane of the blood corpuscles, it will be the concentration of the substance in the fluid phase that determines its effect. Similar conditions are known from the hemolysis process; Leschly (1914) thus showed that when the same amount of erythrocytes is hemolysed in different total volumes the complement concentration will decide how soon complete hemolysis is arrived at. Kiss (1909) and Scheller (1910) also emphasized that the concentration and not the total amount of active substance is of importance to the hemolysis process.

COURSE OF EXPERIMENTS

With a few exceptions human blood was used in all the experiments, drawn by venous puncture and defibrinated by shaking in a bottle with metal coil. As to the risk of a blood-sample considered as "normal" containing specific immune body, see page 94.

The experiments are commenced with washing of the blood corpuscles (if it is not a pure bactericidal experiment). As it is of great importance to know the true concentration of fresh serum in the milieu of reaction the same technique is always used in washing, securing *that the concentration of fresh serum in a spontaneous phagocytosis experiment is less than 1:15,000.*

After the washing the test tubes are prepared, as a rule according to the following schedule:

For a bactericidal experiment the following fluids are mixed in a tube measuring 15×100 mm.:

Fresh serum, diluted or undiluted (obtained by centrifugation of the defibrinated blood for about 10 min. at 2000 revolutions per min.)	0.55 cc.
Saline solution (sterile 0.9 % sol. of NaCl in distilled water) or possible reagents	0.25 cc.
Bacterial suspension (diluted bouillon culture)	0.20 cc.
Total	1.00 cc.

For a *phagocytosis experiment* the following components are mixed in a test tube of the same size:

Washed blood corpuscles with a dry volume of	0.45 cc.
+ the saline sol. required for suspension	0.25 cc.
Serum, saline sol. or possible reagents	0.55 cc.
Bacterial suspension	0.20 cc.
Total	1.45 cc.

Very nearly 1.00 cc. of the total volume in a phagocytosis tube will be free fluid. The hematocrit figures of normal human blood oscillate about 45 per cent., and the influential changes of the composition of the experimental milieu are so great that the actual hematocrit figure need not be considered.

When the test tubes have been prepared so far that only the bacterial suspension is lacking, the suspension or suspensions to be used are made: As a rule the material used is a bouillon culture, about 20 hours old, which is diluted with saline so that the 0.20 cc. added to each tube will contain about 30,000 bacteria (according to the purpose the dose may vary from 10,000 to about 50,000). After addition of the bacterial suspension the actual experiment is started, the rack with all the test tubes being shaken and placed in incubator at 37°.

The time of the experiment is nearly always 30 min., whereupon the reaction is interrupted by the addition to each tube of 9.0 cc. cold saline solution. The cooling and dilution (1:10) obtained by this means produces a very effective interruption of both the bactericidal and the phagocytosis process. After the dilution each tube contains 10.0 cc. fluid which are mixed thoroughly with a Pasteur pipette, whereupon all the tubes (if it is not a pure bactericidal experiment) *are centrifuged simultaneously for 4 min. at 1500-2000 revol. per min.** This slight centrifugation is sufficient to throw down the blood corpuscles completely, and it is now possible from the upper layers of the clear fluid to

* All centrifugations were made in a Stock-centrifuge holding 96 test tubes at a time. The distance from the revolving axis of the centrifuge to the bottoms of the test tubes is 18.5 cm. in the course of centrifugation.

withdraw suitable volumes for inoculation on big agar plates (as a rule 0.1 cc. per plate).

2 or 3 plates are generally inoculated from each tube and as it is necessary to be quite sure that no bacterial division occurs before the inoculation, single experiments cannot be made with more than about 60 plates, considering the lag phase of the bacteria. In large experiments division is safeguarded against by placing the test tubes on water-bath at 5° to 10° during inoculation.

Besides the test tubes used for the actual experiment one control tube per bacterial strain employed is required for each experiment. On culture from this tube, which only contains saline solution and bacteria and is centrifuged in the same manner as the other test tubes, the number of bacteria per experimental dose (0.20 cc.) of the bacterial suspension employed is determined as accurately as possible. The average colony figure determined from the control test tube is termed the *initial value of the experiment*.

The counting of the colonies—i.e. the reading of the experiment—is best performed when the colonies have reached diameters of 1 to 3 mm. If the rapidly growing Breslau strains are used, an experiment finished for instance at 16 o'clock can thus be read next morning.

As to the exact details of the experiments, see the text printed in brevier at the end of this chapter.

ESTIMATION OF EXPERIMENTS

The simplest method is, of course, to state the total of colony figures direct, see for instance Tables 1 and 2. The mean values calculated for the different test tubes are termed *initial*, *bactericidal*, and *phagocytosis* values corresponding to the test tubes from which they originate. On the basis of these values different exponents of the intensity of the bactericidal or phagocytosis processes can easily be computed.

In the case of the bactericidal process the computation is simple: By expressing the bactericidal value in percentage of the

initial value the survival percentage of the bacteria is arrived at direct and this is the natural exponent of the *bactericidal degree*. It should, however, be borne in mind that a *low* survival percentage expresses a *high* bactericidal degree. It must be added that in order to estimate the precision of a given percentage it is, of course, necessary to know the numerical magnitude of the initial value and the number of counts included in the determinations of the initial and bactericidal values respectively.

The aim of such experiments as will be described in the following is nearly always to compare different bactericidal or phagocytosis degrees. As an instance can be mentioned a simple bactericidal experiment comprising an initial value and a number of bactericidal values originating from test tubes with different concentrations of fresh serum. The serum concentration is adapted in the manner that in a given test tube it will always be 60 per cent. of the concentration in the preceding tube. If the different bactericidal degrees or survival percentages are computed and drawn up in a coordinate system, in which the serum concentration is marked down along the abscissa, the S-shaped curve results that is reproduced in Table 1. The resulting curve denotes that the individuals in the bacterial suspension employed are not equally sensitive to alexin, and the shape of the curve gives a graphic representation of the distribution of the sensitivity in the bacterial population (as will be shown in the following this distribution is generally of the type of normal distribution). It follows from the shape of the curve that on comparison between two bactericidal degrees—i.e. on comparison between the alexin activity in two test tubes—it will be necessary to state both of the two survival percentages. It is insufficient just to state the difference between them, which might seem tempting; as will appear from the curve, highly varying differences on the abscissa will, according to the level of the percentage, correspond to a given difference between survival percentages. Only in case the curve in Table 1 had been a straight line—a very special and unbiological case—it would have been justified to use the difference between two survival percentages to denote the dissimilarity between the alexin activity in the corresponding test tubes.

In the case of the *spontaneous* phagocytosis the computation is just as simple as in the case of the bactericidal experiments. Here, too, only one factor—namely the phagocytosis—contributes towards reducing the number of bacteria and, therefore, the natural exponent of the *degree of phagocytosis* is obtained by expressing the phagocytosis value in per cent. of the initial value (see for instance Table 2). After the analogy of the above it ap-

TABLE 1
Titration of the Bactericidal Effect on Dilution.

Total conc. of serum (active + inactive) constant = 55 % in all test tubes.
Conc. of active serum stepwise diminished, (cf. text on p. 27).

This titration constitutes part of the experiment presented in Table 43 (curve a), where, however, the graphic representation is modified by substituting "probits" for percentages. Here and in all the following Tables single determinations from the same test tube are arranged below each other while their mean value is stated next to the last single value separated from this by a semicolon.

Experimental culture: Breslau 206A.

	118	
	124	
	132	
Initial value: $134; 127 \times 2 = 254 (= 100 \%)$		
Conc. of active serum:	Bactericidal values:	Bactericidal degrees:
	10	3.9
17.5 (0.6) ¹ %	19; 14.5	7.5; 5.9 %
	22	8.7
17.5 (0.6) ² %	40; 31	15.7; 12.4 %
	75	29.9
	89	35.0
17.5 (0.6) ³ %	100; 88.3	39.3; 34.6 %
	140	55.1
	150	59.0
17.5 (0.6) ⁴ %	154; 148	60.6; 58.3 %
	191	75.2
	199	78.4
17.5 (0.6) ⁵ %	200; 196.7	78.8; 77.6 %
	225	88.5
17.5 (0.6) ⁶ %	247; 236	97.2; 92.9 %

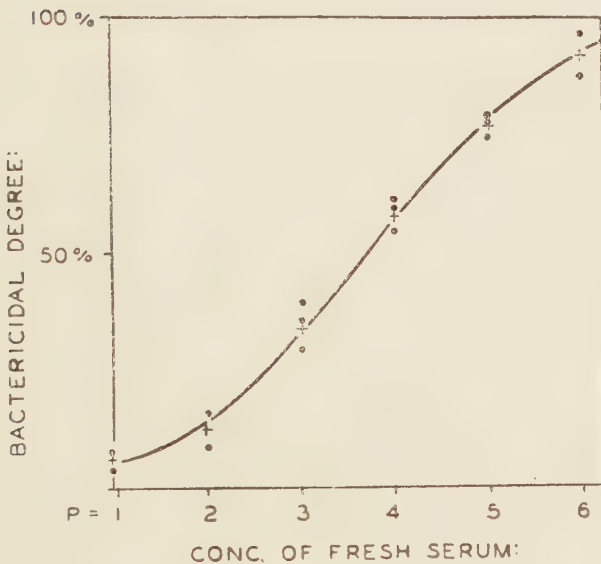
plies, of course, that a *low* percentage of free bacteria means a *high* degree of phagocytosis. As in the case of the bactericidal process, both of the degrees of phagocytosis should be stated in comparison between them, and not only their difference.

In experiments in which bactericide and phagocytosis take place simultaneously the method of computation is less obvious than in the experiments hitherto referred to. If the experimental value is expressed as a percentage of the initial value, just as in the pure bactericidal or phagocytosis experiments, a percentage

TABLE 1 (cont.)

Graphic representation of the titration with the conc. of active serum (given by the exponents p from the first column above) as abscissa and the bactericidal degree (i.e. the survival percentage) as ordinate.

Mean values marked with $+$, single determinations with dots.



expression is certainly arrived at, the magnitude of which depends on the degree of phagocytosis *but not solely on the latter*. The simultaneous course of the bactericidal and phagocytosis processes means that both will contribute towards reducing the number of bacteria.

The simple method of computation, stating the experimental value in per cent. of the initial value, can be used in the cases where only *the total serum activity* is of interest. This applies for instance to the experiment shown in Table 4, the object of which is to examine how the experimental value depends on the duration of the experiment, and to the experiments shown in Table 33A, in which the effects of citrate and oxalate on the total serum activity are determined.

If an exponent of the share of the phagocytosis process in decreasing the number of bacteria is desired, it is, however, necessary to use at least three values for the computation: The necessary figures are—the initial value and bactericidal and phagocytosis values *of the same series*—i.e. values determined from a bactericide and a phagocytosis test tube with identical fluid phases and centrifuged simultaneously. If, on the basis of the three figures stated, the degree of bactericide and that of the total serum activity are computed, two percentage expressions are arrived at from which an impression of the share of the phagocytosis process in decreasing the number of bacteria can be formed. It should, however, be borne in mind that the difference between the value of bactericide and the value denoting the total serum activity does not give the true number of phagocytized bacteria; the simultaneous course of the bactericidal and the phagocytosis processes will involve that a number of bacteria that would have been killed at the end of the experiment may happen to be phagocytized earlier and thus avoid the alexin influence. The true number of phagocytized bacteria will therefore always be $>$ the difference between corresponding values for bactericide and total serum activity.

In some cases it might seem tempting just to use two of the figures stated to compute the degree of phagocytosis and express the latter solely by means of the value denoting the total serum activity re-calculated to per cent. of the bactericidal value. Table

5 shows a special case in which such a simple method of computation is reasonable. In this experiment test tubes with the *same* serum activity but decreasing density of blood corpuscles are compared, and in this case the bactericidal value common to all the test tubes will constitute a new and practical basal value for the computation of the degrees of phagocytosis.

In experiments in which the serum activity is changing from tube to tube (e.g. the titration experiments referred to in Chapter X), the same considerations will, however, apply as those stated in the case of the bactericidal experiments; here, too, it is necessary to state all the experimental values in per cent. of suitable basal values, as comparisons between different sets of degrees of reaction (a set = corresponding values for bactericide and total serum activity) cannot be made without considering the percentage.

For reasons that will be stated in the following the total serum concentration (= the concentration of active + inactive serum) is kept at a constant level in all test tubes in titration experiments. In such experiments it is practical to introduce a special basal value for the computation of the degrees of the total serum activity. The new figure is the phagocytosis value, in a milieu with purely inactive serum (= the spontaneous phagocytosis value) and it represents the limit that will be approached on titration of the total serum activity when—e.g. owing to dilution of the active serum—it will approach nil. The spontaneous phagocytosis value and the ordinary initial value thus form the natural limits for titration of the total serum activity and the alexin activity alone respectively (see for instance Table 43A & B).

In the course of the reference to the phagocytosis process it was implicitly taken for granted that bactericide and phagocytosis are the only factors that will reduce the number of bacteria. It might be feared that still another factor would be instrumental, as the erythrocytes in the test tubes might be imagined to carry along bacteria with them during the centrifugation. In Chapter III, under the reference to experimental sources of error, it will be shown why such effect of the erythrocytes need not be considered.

SUMMARY OF COMPUTATION-TECHNIQUE AND MODE OF REPRESENTATION

In the computations referred to, a total of four different quantities can be considered; i.e. the initial, bactericidal and spontaneous phagocytosis values and values denoting the total serum activity. By "values" is always understood the colony figures read direct.

The intensity or "degree" of reaction is stated as a percentage, the values of reaction read being re-calculated to percentages of the initial value. In the fraction denoting a degree of bactericide or phagocytosis or the degree of the total serum activity the numerator will thus always be the value of reaction read and the denominator will be the initial value; e.g. the *degree* of bac-

$$\text{tericide} = \frac{\text{bactericidal value}}{\text{initial value}} 100.$$

In special cases it may be practical to substitute another basal value for the initial value:

1) An exponent of the degree of phagocytosis in a milieu with fresh serum + blood corpuscles can be formed by re-calculating the value denoting the total serum activity into a percentage of the bactericidal value, see Table 5 where the bactericidal value is common to all test tubes in each of the series of titration and therefore constitutes a suitable basal value.

2) The degree of the total serum activity can be expressed by means of the spontaneous phagocytosis value instead of the initial value as basal figure. The advantages of this method of computation will be seen in titrations of the total serum activity, as reaction values approaching the spontaneous phagocytosis value and not the initial value are found when the serum activity tends towards nil.

On arranging the tables of the experiments it was tried as far as possible to note or take down all the single counts. In certain cases where the differences demonstrated are very great, or in curves where the inclusion of all single determinations would render the figure too obscure, the writer has been content to state the mean values.

In cases where special procedures (especially re-calculation by means of probits) were used in the graphic representation of the results of the experiments, the reason why the special procedure was chosen is stated.

In the following pages printed in brevier the details of the experimental technique are referred to more thoroughly.

DETAILS OF EXPERIMENTAL TECHNIQUE

In most phagocytosis experiments the first phase consists in washing of the blood corpuscles, and as it is of great importance to know the actual concentration of fresh serum in the reaction milieu, as referred to above, the same washing technique is always used: 5 cc. defibrinated blood (i.e. 5 experimental doses of blood) are measured out in one of the ordinary test tubes with a capacity of about 12 cc., when filled completely, and after centrifugation for 10 min./2000 revol. the serum is closely drawn off with a pipette; a carefully squeezed Pasteur pipette removes the last part, however without removing any of the centrifugate. As referred to, all centrifugation is made in a Stock centrifuge holding 96 tubes; it is best not to apply the brake during the washing of the blood, as it involves the risk of the uppermost leukocytes being whirled up again. The blood corpuscles are re-suspended in saline solution, the tube being filled up; the contents are thoroughly mixed with a Pasteur pipette and centrifugation is performed once more, 5 min./1500 revol. now being sufficient as the blood corpuscles are thrown down readily and completely in saline solution. This washing is made 3 times in salt solution and the water is closely pipetted off each time.

In case the serum is of a deep colour colorimetric computation of the serum dilution arrived at can be made by comparing the serum and the first portion of rinsing water. Repeated determinations have shown that the serum is diluted about 1:15 each time the blood corpuscles are washed, i.e. the fluid remaining after the last pipetting is serum diluted 1:15³ (1:3375). It will be seen from the volume of the centrifugate (about 3.0 cc.) and the hematocrit figure (45) that about 25 % of the centrifugate is fluid (3.0 - 45 % of 5.0 = 0.75 cc.). The ultimate serum dilution of the reaction milieu in which, as pointed out earlier, the amount of fluid is fixed at 1.0 cc., (i.e. from $\frac{1}{5} \times 0.75 = 0.15$ to 1.0 cc.) will therefore be about $\frac{1}{15^3} \times \frac{15}{100} =$ about 1:22500, *thus we may safely reckon with a serum dilution of between 1:15,000 and 1:30,000.*

After washing of the blood corpuscles the latter are re-suspended either in saline solution or in specially treated serum. The volume to be reserved for the addition of bacteria and reagents must be considered here. In the centrifugate which contains 5 experimental doses of blood corpuscles (see

above) 0.75 cc. fluid was found, i.e. 0.15 cc. per experimental dose. If 0.5 cc. saline solution is added to the 3.0 cc. centrifugate a suspension is obtained which can readily be sucked into an ordinary 1 cc. pipette; per experimental dose of blood corpuscles this suspension contains 0.25 cc. saline solution, and the maximal rest at disposal will thus be 0.75 cc. (1,0-0,25). So great an extra volume will, however, generally not be required; for instance for the determination of saline phagocytosis only 0.2 cc. will be reserved for the addition of the bacterial suspension, for which reason the 3.0 cc. centrifugate of blood corpuscles are re-suspended in $5 \times (1.00-0.15-0.20) = 3.25$ cc. saline solution and of the resulting 6.25 cc. suspension of blood corpuscles, 1.25 cc. is used for each test tube.

The reagents to be used in the experiment should be prepared in advance by measuring comparatively great volumes from a stock solution (e.g. of citrate); by this means a uniformity and accuracy is obtained that will be of importance, if it is desired to compare a number of tubes, in which the citrate concentration must be constant whilst some other factor is varied. If a citrate concentration of 0.3 % is desired, 1.4 cc. saline solution is for instance mixed with 0.6 cc. 10 % Na-citrate solution and 0.1 cc. of this mixture is added to each tube.

The bacterial suspensions that have to be added are prepared in the following manner:

According to the density of growth 0.02 to 0.05 cc. of a bouillon culture is added to a test tube containing 2.0 cc. saline solution, the tube is corked and shaken well. 0.1 cc. is transferred from the dilution tube to a bottle with 10 cc. saline solution and the bottle is thoroughly shaken (for about 1 min.).

This procedure allows the preparation of dilutions of several cultures in a fairly short time, which is of special importance in case the bacteria used cannot stand suspension in saline solution for any length of time without diminution of the percentage of germination. (The Breslau strains most frequently employed in the experiments, however, resist the ordinary saline solution well; cf. p. 38).

The bacteria are transferred direct from the dilution bottle to the test tubes—the dose generally used is 0.2 cc. containing 10-50,000 bacteria, (the density of growth in the bouillon culture being about $1.5-2.5 \times 10^9$ per cc.). The bacterial suspension is added with a well pointed 1.0 cc. pipette and this measuring, which is the most decisive, is made as exactly as possible, the point of the pipette being put down alongside the wall till immediately over the surface of the fluid in the test tube that is held obliquely (cf. p. 63). After the addition the bacterial suspension is lying uppermost as a *fairly well defined layer* in the test tube, and now the experiment is started by shaking the rack with all the tubes and placing it in an incubator at 37°.

The duration of the experiment is generally 30 minutes, during which the rack is shaken once; this is sufficient and increased phagocytosis is not

obtained by placing the tubes in a shaking device in the incubator. On the contrary, in the case of some bacteria, shaking seems to render the "binding" between leukocyte and bacterium difficult. A "Kahn" shaker which with a swing of 4 cm. makes 150-200 strokes per minute was used for the experiments.

It appeared that in inactive milieu the phagocytosis is independent of the temperature within the interval from 10° to 37° (see Table 2); spontaneous phagocytosis determinations can therefore be made at room temperature, which is of importance to some saline-phagocytosis experiments, the bacteria being better able to stand suspension in saline solution at about 20° than at 37° (cf. p. 39).

The experiment is interrupted by rapid dilution of the fluid in each tube with 9.0 cc. cold (4° or 5°) saline solution; by this means blood corpuscles and bacteria are distributed in a large space and at the same time the bactericide- and phagocytosis-promoting processes are greatly inhibited by dilution and cooling. (As to the importance of the slight difference there may be between the length of the experimental period for the first and the last treated tube see p. 38).

After the dilution the contents of each tube are mixed by moving a Pasteur pipette up and down the tube, sucking it full and emptying it 50 to 75 times; 25 to 50 cc. thus pass the pipette and experience shows that sufficient homogeneity is obtained, cf. p. 61. In order not to delay the experiment it is practical to have an assistant ready to mix as the tubes are being filled.

Before the plates are inoculated all the test tubes are centrifuged, unless it is a pure bactericidal experiment. 4 min. and about 1500 revol. will be sufficient to give a completely clear fluid (in this centrifugation the brake of the "Stock"-centrifuge is applied as only the uppermost layers of the fluid

TABLE 2
*Saline Phagocytosis at Different Temperatures, with and without
Mechanical Shaking.*
Experimental culture: Breslau 206.

Initial value	Values and degrees of saline phagocytosis at		
	37° + shaking	37° — shaking	10° — shaking
113			
114	36	43	35
136; 121	44; 40	46; 44.5	40; 37.5
100.0 %	34.0 %	37.2 %	31.4 %

Comparison of the mean values 40, 44.5 and 37.5 gives $P = 82\%$, (the same 0.1 cc. pipette used for withdrawal from the three tubes).

Formulae for computation of "t"- and "P"-values will be found on p. 45.

are to be used). In this connection, too, it is advantageous that the experiments are interrupted by dilution: It is unnecessary to consider the slight differences which after the dilution remain between the fluid phase in control tubes and in test tubes varying *inter se*; greater differences of specific gravity would cause the bacterial density during centrifugation to be influenced *differently* in the test tubes, and the comparability would thus be compromised; another advantage is that it is possible several times to take fluid for inoculation from the column, which after dilution is 8 cm. in height, without getting down in really deeper layers. In the course of centrifugation the number of bacteria in the uppermost layers will as a rule only decrease by 10 to 15 %, and therefore at any rate 10×0.1 cc. can be taken for inoculation without the effect of the centrifugation on the bacterial density becoming noticeable.

To determine the bacterial density, inoculation on plates of a given volume is used: On a big agar plate (13 cm. in diameter) 0.1 cc. fluid is spread with an angular, flamed glass-rod. The fluid is taken from the uppermost layer in the test tube with a 0.1 cc. pipette with rubber stall for suction; with some practice it is possible to measure almost as accurately in this manner as with a mouth pipette—cf. p. 62. It is important that the agar plates have a smooth surface and are suitably dried so that the drop of fluid dries fairly rapidly, and still not until it has been evenly spread.

Plates of the following composition were used for all experiments with Breslau strains:—

Placenta agar, 1 liter (pH 7.8-7.6).

Bromthymol blue, 1: 500 40 cc. + 1g $\text{Na}_2\text{S}_2\text{O}_3 \cdot \text{H}_2\text{O}$.

Lactose-saccharose sol. (ää 33 %) 35 cc.

Crystal violet (1 ‰) 5 cc.

Owing to the difficulties of getting supplies a number of the experiments were made with plates in which the substitute "Litex" was used instead of agar; there was no noticeable difference in the percentage of germination on the two kinds of plates, and it was always easy to distinguish the characteristic salmonella colonies from possible contamination.

The plates employed do not, however, offer optimal conditions of growth, although the addition of crystal violet only inhibits the growth of the bacteria of the salmonella group slightly. It should be borne in mind that thus it is not the actual number of surviving or free bacteria that is determined but the number that is able to grow under the given conditions. In a few cases this may lead to misinterpretation of an experiment. If for instance a suspension of bacteria in fresh serum is diluted after a suitable period (experiment with preliminary treatment), different figures for the surviving bacteria may be found, if the dilution is made both in saline solution and in a suitable liquid culture medium. From the first suspension the number of bacteria that are sufficiently vital to grow after direct transmission to agar surface is determined; from the suspension in liquid culture medium, where the most attenuated bacteria have regenerated somewhat,

figures are found, on the other hand, which are nearer the actual number of living bacteria.*

Another consequence of these considerations is that, strictly speaking, plates made simultaneously and of the same agar mixture should always be used for the same experiment.

To determine the exact number of bacteria one control tube per culture employed is required for each experiment: 9.8 or 9.9 cc. saline solution is measured off in a test tube and to this is added 0.2 or 0.1 cc. bacterial suspension from the dilution bottle; the contents of the control tube are mixed *and centrifugation is performed as described above*. In experiments where, owing to bactericide or phagocytosis, the number of bacteria is expected to be greatly reduced, it is convenient to start from a relatively high figure, which is best determined by means of half a dose (0.1 cc.) so that the growth is not too dense on the control plates. It is convenient to deal with the control tubes and the inoculation on plates from these (as a rule 2 plates per tube) while the rest of the test tubes are in the incubator. This limits the number of different cultures to be dealt with simultaneously; within half an hour 10 control tubes at the utmost can be handled and inoculations made from them.

The periods and the extents of experiments which it will be possible to use will first and foremost be determined by the length of the lag phase of the bacteria employed. If it was necessary to reckon with the possibility of bacterial division in the course of the experiment, control tubes would be required throughout the experiment, and the conditions of growth in control and test tubes would have to be compared in a preliminary experiment.

When the cultures are 16 to 24 hours old the Breslau strains employed have a lag phase of about 1 hour—i.e. about the same as K. A. Jensen (1927) found for ordinary coli strains on direct microscopy of bacteria on agar-block. The experiment, Table 3, shows how 16 and 24 hours old cultures in bouillon are still at rest after 45 min.; the 16 hours culture seems to be growing after 90 min., whereas an 8 hours culture displays growth already after 45 min.

As already referred to, the bactericidal and phagocytosis experiments are interrupted by dilution and cooling down; besides stopping the reaction an increase of the lag phase of the bacteria is obtained, and repeated control counts have shown that when a low temperature is kept (in large experiments a cold waterbath is used) no bacterial division occurs for the first 2 hours after the beginning of the experiment. The consequence is that a little more than 1 hour can be used for inoculation, and experiments with about 60 plates can thus be performed without any risk.

* These special considerations are, however, of a more theoretical interest, and the differences that can be found are very slight.

There are still two factors which might limit the size of the experiments:

- 1) The time required for interrupting an experiment and
- 2) —in determination of saline-phagocytosis—the tolerance of the strains used to saline solution.

re 1) It will be seen from the experiment, Table 4, with determinations at different times, that after 30 min. the phagocytosis and bactericidal processes are fairly well advanced; this means that the time required for interrupting the experiment will be numerically of comparatively slight importance—with 25 test tubes it is 4 to 5 minutes at the utmost (cf. Table 4 and p. 41). Moreover the maximal 5 minutes' difference in time of the experiment will never be reached, for in large experiments care will be taken to interrupt in the sequence that tubes that have to be compared direct are dealt with immediately after each other; in long series of titration the time error will thus be evenly distributed over the whole series. As to the time course of the bactericidal process, which is quite similar to that of the serum phagocytosis process, see Table 47, Chapter XI.

re 2) In determination of saline-phagocytosis the experimental milieu only contains blood corpuscles and bacteria in saline solution (actually serum-saline 1: about 20,000, a solution which, however, does not promote phagocytosis and has none of the properties of culture media), therefore it will be necessary to make sure in advance that the bacteria employed will be able to stand suspension in saline solution during the experiment without decrease of the percentage of germination. In the case of a few Breslau strains (the O-forms) it applies that suspension in saline solution at 37° leads to moderate killing of the bacteria in the course of 45 to 60 min.; at room

TABLE 3

Determinations of the Lag Phase in Breslau Cultures of Different Age at 37°.

Culture A: Breslau 1, bouillon culture 24 hours old.

„ B: „ 1, „ „ 16 „ „ .

„ C: „ 1, „ „ 8 „ „ .

This experiment was carried out by P. Krag with a special technique permitting withdrawal of and inoculation with small, well defined volumes.

Culture	Time of withdrawal		
	0 min.	45 min.	90 min.
A	5	9	8
	7	9	13
	14; 9	12; 10	16; 12
	8	9	15
B	10	10	16
	13; 10	12; 10	16; 16
	6	11	23
	7	20	27
C	17; 10	22; 18	40; 30

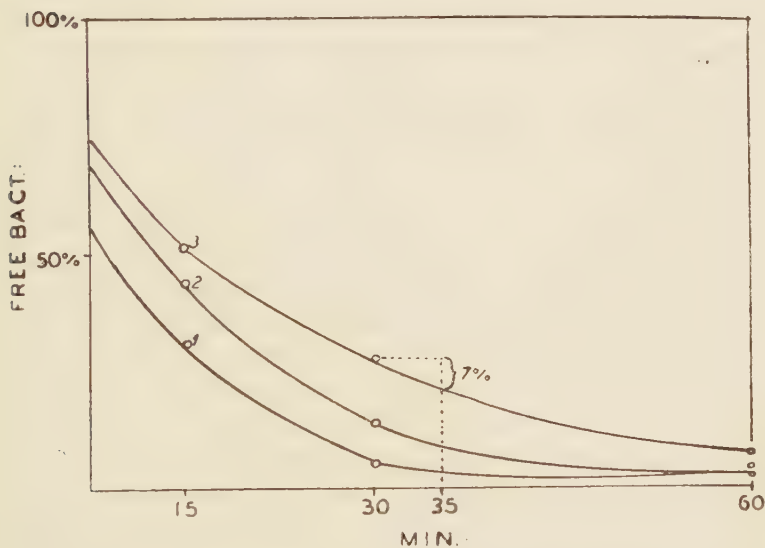
temperature, on the other hand, the percentage of germination in saline-suspensions of all the Breslau strains examined is constant for 60 to 90 min. As referred to above, the degree of saline-phagocytosis is independent of the temperature and, therefore, such experiments should be performed at room temperature, where the percentage of germination is sure to be constant till the inoculation can be made.

The reading of the experiments is made by direct counting of colonies, and this is most safely done when they have reached a size of 1 or 2 mm. on the most crowded plates. A "Breslau" experiment finished at about 16 hours can thus be read next morning.

Plates with up to 400 or 500 colonies are easily counted with a counting watch (though it is not convenient with too many counts over 300). The plate is divided in suitable rectangles and now and again a control is made by double counts. The error which might be feared owing to confluence on crowded plates is very slight under the given conditions, as shown by Krag; when for instance the number of colonies rises from 180 to an expected value of 300 the loss through confluence averages less than 2 %.

TABLE 4

Determinations of the Degrees of Total Serum Activity after 15, 30 and 60 Min. in Different Experimental Milieus.



Curve 1: 55 % fresh serum.

" 2: 55 % " " with slightly inhibited activity.

" 3: 55 % " " more strongly inhibited activity.

Experimental culture: Breslau 1.

CHAPTER III

CAUSES OF VARIABILITY AND ACCURACY OF EXPERIMENTS

Before the more ordinary causes of variability are mentioned it will be necessary to deal with three points that are of fundamental importance to the experimental technique and which all of them might be imagined in advance to give rise to gross systematic errors:

a) Can we be sure that the number of bacteria remains constant in the course of inoculation, which, as referred to, lasts for about 1 hour in large experiments? The tolerance of the bacteria to saline solution and the risk of bacterial division have been mentioned; it is, however, also necessary to make sure that in the case of the bactericidal process there will be no "after-effect" similar to the oft-mentioned "after-hemolysis".—The question was examined in a series of experiments, cultures having been made from the same test tubes for bactericidal experiments both early and late in the course of the inoculation period. All these control counts showed that within the first hour—i.e. for so long a time a test tube may be allowed to wait, considering the risk of bacterial division—the number of bacteria remains constant.

A special examination of this question with combined determinations of the bactericidal degree and the degree of the phagocytosis influence in the period after the cessation of the alexin and opsonin effects is described on page 110 and in Table 30. *This experiment shows that both the percentage of survival and the phagocytability remain constant within the first hour after the interruption of the experiment.* The reason why this important examination is only referred to in full later on is that

the experiment (Table 30) requires a special technique of preliminary treatment that is described on pages 107-109.

b) By what right do we take the liberty to draw conclusions as to the reaction intensity from *one* determination made in the course of a process that must be supposed to last a long time? It is described on page 38 how both the bactericidal and phagocytosis processes seem to take a continuous course with constantly decreasing values of surviving or free bacteria till bacterial division occurs. This fact justifies an estimation of the reaction intensity on the basis of a single determination, which must be made at a proper moment where the difference between strong and weak reactions is distinctly seen. The 30 min.-experiment complies fairly well with this requirement and at the same time the processes taking a rapid course are so far advanced after 30 min. that slight inaccuracies in the delimitation of the time of the experiment exercise no great influence on the comparability between the single test tubes. The importance of exact time of the experiment for all the tubes is dependent on the steepness of the time curves when the experiment is interrupted and will, therefore, vary with the rate of reaction. What has to be avoided—and also is avoided in the 30 min.-experiment—is to interrupt the experiment at a time when the curve may be very steep.

c) The third fundamental question was referred to already in the last chapter; it is the question whether the erythrocytes—or more correctly all the formed elements of the blood—may be considered mechanically indifferent in the course of centrifugation. As will appear from experiments in Table 5, the number of blood corpuscles must be diminished to about 1 per cent. of the normal, when the phagocytosis is marked, in order to reduce the latter to a minimum. It has proved very difficult to reduce the number of leukocytes sufficiently without influencing the density of erythrocytes; the experiment in Table 7, in which the leukocyte film was pipetted off the surface of the centrifugate twice in the course of the washing of the blood corpuscles, only gave a reduction of the leukocyte figure of about 40 per cent. for which reason it was considered impossible to obtain a reduction of the leukocyte figure by this method great enough to minimize phagocytosis.

It is, however, possible indirectly to estimate the importance of the formed elements in the course of centrifugation. Among the observations that will be described later on there are two indicating that in the course of centrifugation no bacteria are carried down by erythrocytes and that bactericide and phagocytosis, therefore, are the only factors causing a reduction of the number of bacteria, as presupposed in the description of the estimation of the experiments. The two observations are:

1) Among the Breslau strains examined there is the motionless O-form, as already referred to; this strain is characteristic in that it is not phagocytized at all in saline milieu, though distinctly sensitive to alexin and opsonin in suitable concentrations. The experiment represented by Table 39, in which a series of corresponding values of bactericide and phagocytosis was determined with the Breslau O-form, includes control determinations of the saline-phagocytosis and, as will appear from the Table, *the initial and saline-phagocytosis values are identical*. As far as the Breslau O-form is concerned the formed elements of the blood have thus caused no mechanical removal of bacteria in the course of centrifugation.

2) It will appear from the inhibition experiments described in Part II that when a phagocytosis process is inhibited through influence on the bacteria either by a normal or specific antibody, a complete discontinuation of all phagocytosis is obtained in some cases, the phagocytosis value thus becoming identical with the initial value.—See Table 17, in which the spontaneous phagocytosis has become practically discontinued by inactive serum in a concentration of 55 per cent., and Table 27, in which both the spontaneous phagocytosis and the serum phagocytosis are discontinued completely on addition of specific immune body.—The inhibition experiments referred to, performed with the normal, movable Breslau strains, thus seem to indicate that the formed constituents of the blood do not bring about any mechanical removal of bacteria in the course of centrifugation as far as the normal strains are concerned either.

The causes of variability that otherwise must be considered in measurements of this nature have been thoroughly studied by

Krag, both theoretically and experimentally. Krag's, as yet unpublished data show that 3 well-defined domains of variability must be considered:

1) Variations owing to *random* heterogeneity of the experimental milieus that have to be compared.

2 a) Variations in the withdrawal and measuring when fluid is pipetted for inoculation.

2 b) Variations in the withdrawal and measuring when bacteria are distributed to the test tubes from the mixing bottle.

By variation in the withdrawal is understood the variability that is dependent on the degree of homogeneity in the bacterial suspension from which the withdrawal is made; this variability must *a priori* be expected to be of the "Poisson-type", i.e. there is direct proportionality between the colony mean K and the variance (or square of standard deviation) $V\{K\}$.

The variation in measuring is the variability in measuring the volume added to the test tube or used for inoculation.

re 1) The inevitable milieu variations have been estimated for each of the factors concerned (bacteria, serum, blood corpuscles, and the different reagents added). The most important result of these examinations is *that direct comparison between the reactions in different test tubes is only permissible within the same experiment*. Quantitative comparisons between results from different experiments proved to be difficult, several of the reagents employed varying quite considerably; this applies especially to the bacteria, whose alexin- and opsonin-sensitivity depends largely on the method of culture, and to serum, whose activity and sensitivity to different reagents may also fluctuate.

In view of these variabilities the experiments reported in the present publication were designed in such a manner that all quantitative comparisons (especially titrations) were made within the same experiment.

re 2) The accuracy with which the bacterial density in a test tube is determined by 2 or more single counts was examined on the basis of a large part of the total experimental material selected at random. First it was shown that we may reckon with the same variation of withdrawal from all test tubes, independently

of differences in the composition of the milieu and in the treatment the contents were given. Then all duplicate determinations have been compared, the object being to examine whether all differences could naturally be considered due to the fact that the bacteria in all test tubes are distributed in a common distribution (possibly a normal distribution). It appeared that, after exclusion of a few experiments in which there were conspicuous accumulations of duplicate determinations with large differences, the material could be considered homogeneous.

The result of the examination was that the variation in the withdrawal can be considered to be of the Poisson-type with a variance (s^2) almost equal to the colony figure (K) multiplied by the empirically determined constant 1.1.

Determination of the variation in the withdrawal and measuring from the mixing bottle then led to a combined expression for the variability with which we must reckon when comparing colony means determined from two different test tubes. As it has been impossible to compute the most important term of the expression for the total variability with accuracy (the variability in the withdrawal from the test tubes) it was preferred to determine an upper and a lower limit of the total variability; these limits can be expressed by the following two estimates of the variances (s^2):

$$\begin{aligned}s^2_{\max.} &= 1.2K + (0.01K)^2 + (0.02K)^2, \text{ and} \\ s^2_{\min.} &= 1.0K + (0.01K)^2 + (0.02K)^2, \text{ where K is the mean}\end{aligned}$$

of the colony figures and the two squares represent the variability in measuring the volume of bacterial suspension added to the tubes and the variability of the pipettes used for the inoculation respectively.

In practice $s^2_{\max.}$ should be employed when it is desired to demonstrate a significant difference between two colony means, whereas $s^2_{\min.}$ should be employed when two means are to be regarded as identical.

When evaluating differences or identity between two mean colony figures, determination of a "t" has been used in the present publication. Rasch has stated the following formula for t, in which it is considered on how many single counts the de-

terminations of the two colony means (K_1 and K_2) that have to be compared are based:

$$t = \frac{K_1 - K_2}{\sqrt{s^2 \left(\frac{1}{a} + \frac{1}{b} \right)}}, \text{ } a \text{ and } b \text{ being the numbers of single}$$

determinations used for calculating K_1 and K_2 respectively, and s^2 being computed from the weighed mean figure of K_1 and K_2 :

$$\bar{K} = \frac{aK_1 + bK_2}{a + b}. \text{ For computing } s^2 \text{ the exponent of } s^2_{\text{max.}}$$

or $s^2_{\text{min.}}$ is chosen according to the object of the examination.

The t determined by the above formula is subject to some uncertainty owing to the fact that $\bar{K}$ cannot be determined accurately; this means that the uncertainty of t depends on the number of single determinations used for determining $\bar{K}$, and that the uncertainty is greatest when the K -values are small. If it is required to determine $\bar{K}$ with 95 % confidence, the following limits of t can be computed, applying to comparison between 2 duplicate determinations:

$$\begin{array}{llll} \bar{K} = 25 & \text{gives the limits } t \pm & \text{about } 10 & \% \\ \bar{K} = 100 & \text{,, , , } & t \pm & \text{,, } 5 \% \\ \bar{K} = 400 & \text{,, , , } & t \pm & \text{,, } 2.5 \% \end{array}$$

With increasing number of single determinations used for $\bar{K}$ the limits are narrowed in direct proportion to $\sqrt{a+b}$.

In cases where 3 or more mean colony figures are supposed to be identical, an evaluation of all single determinations in one was made instead of determining t -values for all combinations: On the basis of the common mean figure, $\bar{K}$, the sum of the squared deviations from the mean has been computed and on division by the theoretical s^2 ($s^2 = 1.1\bar{K} + (0.01\bar{K})^2 +$ possibly $(0.02K)^2$) the corresponding χ^2 -value has been determined. The final result is stated by the probability "P" corresponding to the χ^2 found, "P" being given in per cent.

In order to convey an impression of the rôle played by the different parts of the expressions for s^2 and t the calculation of t for a single experiment is referred to in detail on page 64. A direct impression of the figures with which we generally have to operate and the differences occurring between determinations from the same test tube is best obtained from Tables 1, 6, 16, 20, 24, 29, and 30, in which numerous duplicate and replicate de-

terminations are collected. In the following text in brevier the different considerations regarding the errors are gone through more thoroughly; the grouping refers to the above groups of errors 1, 2a and 2b.

VARIATIONS OF MILIEU (1)

The variations of milieu must be dealt with from 2 different points of view.

a) Variations that may become significant sources of error within the same experiment, and

b) Variations that will only become significant when different experiments are compared.

The following milieu factors will be referred to in detail: Bacteria, serum, blood corpuscles, and "additional" reagents.

Bacteria: Within the same experiment we work with a definite bacterial population, and as far as bacteria are concerned we are not subject to other errors than those of pipetting and withdrawal (see 2a and 2b). It must, however, be strongly emphasized that the reaction of a given strain of bacteria to active serum and leukocytes is not constant, and that fluctuations of degrees of bactericide and phagocytosis need not correspond to fluctuations of morphology or antigen structure as determined by absorption and agglutination.

It was very tempting to take up some of the questions in connection with this special variability in one and the same strain of bacteria; but it has only been possible to do so after the work with the fundamental questions to be dealt with here had been concluded.* From the experience gathered later on about variations of sensibility a simple and fairly safe way of procuring comparable bacterial cultures for phagocytosis experiments covering a longer period will be pointed out in this connection: The cultures are prepared from stab-cultures in extract-agar, and the 1st bouillon-generation from the same stab kept at $+4^{\circ}$ is used for experiments that have to be compared.

Serum: As referred to on p. 34 the preparation of the different reagents (including serum dilution and bouillon suspension) is made by measuring comparatively large volumes, from which the distribution is made to the test tubes. In case small amounts of fluid must be measured, 0.2- or 0.1 cc.-pipettes are always used. The variability resulting from careful use of a mouth pipette depends on the volume measured. The standard deviation does not

* These questions will be reported on in a subsequent paper.

exceed 2 % of the volume, however, and is less when well-pointed pipettes with long scales are chosen (the pipettes should be chosen so that the volume desired is measured at once—as the variation of course increases with each measuring, see also p. 63).

The different titration experiments have shown that the serum concentration—even in the most sensitive interval—must be altered by 5 to 10 % to give safe alterations in the percentage of surviving or free bacteria, i.e. the variations in the distribution of serum to the test tubes are very slight as compared to the alterations of the serum concentration that are of importance to the result of the experiment (see for instance Table 1).

It is quite another matter if different experiments have to be compared. Browning & Mackie (1914) have shown that when serum is kept at -8° its normal antibacterial activity is kept unaltered for a long time; this observation has been fully confirmed, and serum kept in a frozen condition can therefore be used to carry through series of comparable bactericidal experiments. Before refrigeration the serum for such series of experiments should be distributed to several test tubes, a fresh tube being used for each experiment as repeated thawing may impair the serum activity. Moreover the serum should always be prepared with the same technique; Walker (1903), Henderson-Smith (1906), and Morrison (1922) thus describe that there is a considerable difference between the activity of serum obtained through centrifugation of defibrinated blood and serum obtained through the spontaneous retraction of the clot.

More special methods of preservation—e.g. the addition of 4 % NaCl stated by Friedberger (1907) or vacuum drying of serum—are either inapplicable or cumbrous in this connection.

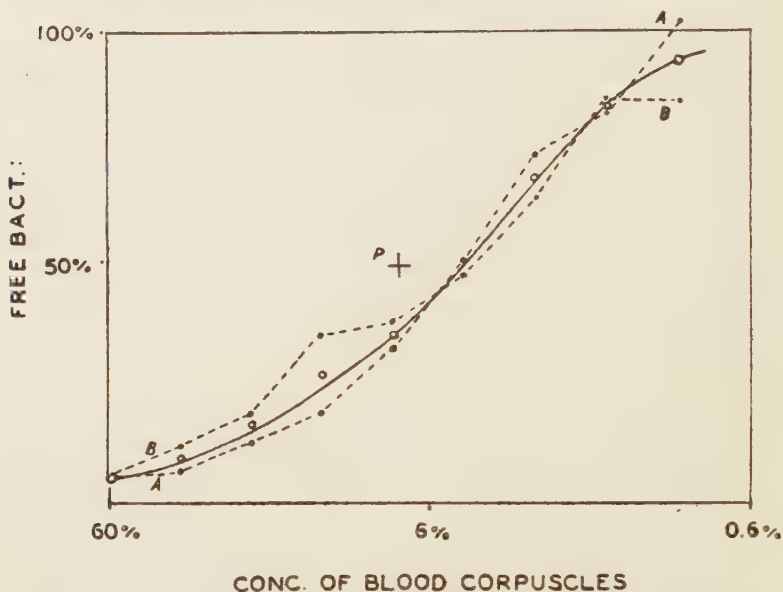
Owing to the limited keeping qualities of the blood corpuscles (see below) it is, however, necessary to draw fresh blood every other day for series of phagocytosis experiments. The question therefore nevertheless arises which fluctuations the serum activity passes through. Experiments performed throughout a long period have shown that the same healthy donor yields serum with practically unchanged activity for months. If series of experiments have to go on for a longer period, and especially if several donors are used, it will be necessary to link up the experiments by keeping (freezing down) a little serum from each experiment and using it as a control on the next day of experiment in order to compare its activity with that of the new portion of serum. The increase of the variability determined from single experiments which theoretically must be expected in this coupling up will not be referred to here, as such long series of comparable experiments have not been used in the present work.

Blood corpuscles: As regards the blood corpuscles, too, it applies that within one experiment the comparison can be made at once, as the small changes of the density of the blood corpuscles that may be due to the variability in measuring are without any importance (cf. Table 5).—In

comparison between different experiments we must, on the other hand, reckon with a risk of both quantitative and qualitative fluctuations in the composition of the population of blood corpuscles. In the same healthy donor both the quantitative and the qualitative variations are slight, at any rate when the blood samples are drawn at the same time of the day. This is shown by the fact that for instance a saline-phagocytosis-percentage can be reproduced with great certainty when the same donor is used and the

TABLE 5

Titration of the Degree of Phagocytosis with Stepwise Diminution of the Density of the Suspension of Blood Corpuscles and Unchanged Serum Activity.



The two experiments performed simultaneously; mean values marked with o.

The computations of the degrees of phagocytosis based on the amount of surviving bacteria in control tubes without blood corpuscles, (bactericidal controls, see text on p. 30-31).—The density of the suspensions of blood corpuscles stated in per cent. of the normal density.—P marks the crossing point between the 50 %-level and a symmetrical, S-shaped curve with the same extreme values as the experimental curve, (cf. text on p. 49).

Curve A: Initial value: 299; bactericidal control: 233 (77.9 %).

Experimental culture: Breslau 1.

„ B: Initial value: 185; bactericidal control: 94 (50.8 %).

Experimental culture: Breslau 206.

blood is drawn at the same time (see Table 7); it appears, however, also from the experiment that there may be a fairly considerable difference between saline-phagocytosis percentages determined with blood corpuscles from different donors.

It applies to the same blood sample that only fairly great changes of the total density of blood corpuscles influence the degree of phagocytosis to any extent worth mentioning; this appears from simple dilution experiments in which the composition of the fluid phase and the total volume are kept at a constant level whilst the number of blood corpuscles is reduced. As shown in Table 5 the experiments, performed with two Breslau strains of different sensibility, give a common, asymmetrical, S-shaped curve with a faint bend at the beginning and a more marked bend towards the end, (as referred to on page 30, the phagocytosis degrees in this experiment alone are given as phagocytosis values expressed in per cent. of the common bactericidal value).

In connection with these experiments the writer tried theoretically to evolve the interdependence between leukocyte figure and phagocytosis degree on the basis of considerations of the chances of interference between bacteria and leukocytes. The computations, which in spite of theoretical interest can hardly be of great importance to the present work, will not be dealt with here, especially as the writer did not succeed in arriving at safe results. In the discussion in Chapter X about the importance of the chances of interference to the shape of the titration curves a reference is therefore made to the experimentally established interdependence, between leukocyte figure and phagocytosis degree in Table 5.

It might be imagined that the erythrocytes, and possibly the thrombocytes, played a rôle in the reaction, either indirectly by influencing the possibilities of motility and contact in the milieu or by taking down bacteria in the course of centrifugation. In order to get a picture of the importance of the isolated leukocyte variation, experiments were made in which, as far as possible, only the leukocyte figure is varied: By means of a drawn-out Pasteur pipette, the point of which was bent in U-shape, the leukocyte film on the surface of the centrifugate was removed from below. This was done twice in the course of the washing of the blood corpuscles, and phagocytosis experiments were made with blood samples in which leukocyte and erythrocyte figures were determined. It will appear from Table 6 that the degree of phagocytosis decreased with the leukocyte figure in a manner corresponding fairly well to the variation in the experiment, Table 5, where the number of formed elements is reduced simultaneously. The experiment in Table 6 was performed with the very sensitive strain, Br. 206 A (see page 150) and rather slight serum activity. It can be roughly computed from the titrations with the same culture referred to later on (Table 43 A) what the phagocytosis percentages in Table 6 correspond to expressed in percentages of the surviving bacteria, and from Table 5 the densities of blood corpuscles corresponding to the percentages thus re-calculated can be read. Instead of

22.8 % and 35.6 % about 45 % and 57 % are found on re-calculation and according to Table 5 blood corpuscle concentrations in the ratio 1:1.5 correspond to these figures, i.e. very nearly the same ratio as between the leukocyte figures on Table 6 (8,000:13,100).

Still, this comparison between phagocytosis degrees in an isolated part of the field of reaction does not give any decisive reply to the question of the mechanical importance of the formed elements and, as referred to on page 42, it is indirectly that we get the best support of the assumption that the blood corpuscles do not take down unphagocytized bacteria in the course of centrifugation.

The keeping qualities of the blood corpuscles, especially of the leukocytes, is limited. As pointed out by Lambotte & Stiennon (1906), among others, the washed blood corpuscles, when kept at $+4^{\circ}$, can, however, be used after 24 hours, the activity then being unchanged (see Table 7); this is of importance where serum has to undergo lengthy preparations (e.g. absorption or heat treatment) so that preparations and phagocytosis experiments cannot be carried through in the course of one day. It is, however, not advisable to store the blood corpuscles for more than one day.

It will appear from the statements about the keeping qualities of blood corpuscles that phagocytosis experiments performed at intervals of several days cannot be linked up like pure bactericidal experiments. Instead of this a known bacterial culture derived direct from stabs must be included in each new experiment, and the degree of phagocytosis to this must be determined as control.

In connection with the reference to the blood corpuscles some control experiments will lastly be shown proving that the washing procedure does not reduce the capacity of the leukocytes to "bind" bacteria (Table 8), and

TABLE 6

Two Phagocytosis Experiments with Identical Fluid Phases but with Different Numbers of Leukocytes.

I. Normal density of leukocytes.

II. Reduced density, (cf. text on p. 49).

Initial value: 675. Experimental culture: Breslau 206A.

Density		I	II
Values and degrees of phagocytosis:		151	244
		154	252
		157; 154	228; 241
		22.8 %	35.6 %
Blood cell counts:	Erythrocytes:	4.56 mill.	4.38 mill.
	Leukocytes:	13100	8000

TABLE 7

Saline Phagocytosis determined with Blood Corpuscles from Different Donors or of Different Age.

a) Washed blood corpuscles from different donors: I ctr. II, and III ctr. IV.

b) Washed blood corpuscles from the same donor, blood samples drawn on different days: IV ctr. V.

c) Washed blood corpuscles from the same donor used 3-4 and about 28 hours after the blood sample was drawn: V ctr. VI.—The suspension of blood corpuscles was stored at $+ 4^{\circ}$.

Experimental culture	Experiment number	Donor and date	Initial value	Values and degrees of saline phagocytosis
Breslau 1	I	Donor R 2/3 1944	89	
			89	74
			83	82
			105; 91.5×2	93; 83
			183	45.6 %
Breslau 206	II	Donor M 29/2 1944	243	278
			228	286
			242; 238 $\times 2$	342; 306
			476	64.3 %
Breslau 206	III	Donor R 2/3 1944	103	
			110	
			126	45
			134	61
			146; 124 $\times 2$	61; 56
Breslau 206	IV	Donor M 29/2 1944	248	22.6 %
			122	75
			128	83
			141; 130 $\times 2$	85; 81
			260	31.2 %
Breslau 206	V	Donor M blood drawn 21/2, used 21/2	90	58
			91; 90.5×2	61; 59.5
			181	32.9 %
Breslau 206	IV	Donor M blood drawn 21/2, used 22/2*	96	62
			105; 100.5×2	78; 70
			201	34.8 %

* It has, however, several times been found that the phagocytosis produced by an 24-30 hours old suspension of washed blood corpuscles was a little stronger than that produced by a fresh suspension.

Very nearly all the experiments referred to in the present work were carried out with blood from one of the two donors mentioned above.

it must be mentioned that in all the following experiments homologous sera and blood corpuscles have been used (apart from anticomplementary serum, and rabbit immune sera, both of which are, however, as a rule used in very slight concentrations).

In the description of the experimental technique it was stated that when preparing bacterial suspensions for the experiment the density of growth in the bouillon cultures is considered. It was also stated that the bacterial dose may be adjusted according to the character of the experiment (a great dose in experiments where a marked reduction of the number of bacteria is expected). This arbitrary adjustment implies that even considerable changes in the ratio between leukocyte figure and amount of serum on the one hand and number of bacteria on the other hand play no part.

As to the relation between serum and bacteria Hahn (1895) and Hektoen & Ruediger (1905) pointed out that at a given serum concentration the dose of bacteria is of importance to the result of a bactericidal experiment. This dependence is not found when we work with so small amounts of bacteria and in experiments of short duration; the experiment (Table 9), in which the same percentage of survival occurs with "normal" and $8 \times$ normal bacterial dose shows that the latter may freely be varied far beyond the limits that must be fixed out of regard to counting. (The said experiment,

TABLE 8
Comparison between the Phagocytic Power of Leukocytes treated in Different Ways.

Mixture a: Defibrinated blood, + 0.3 % citrate (as to the part played by citrate see chapter IX).

Mixture b: Defibrinated blood centrifuged three times as during the usual washing procedure, the blood corpuscles each time being resuspended in the supernatant serum; + 0.3 % citrate.

Mixture c: Blood corpuscles washed three times in saline solution and resuspended in fresh serum, + 0.3 % citrate.

Experimental culture: Breslau 1.

Initial value	Experimental values and degrees		
	Mixt. a	Mixt. b	Mixt. c
345	55	67	57
329	50	69	50
337; 337	55; 53	71; 69	61; 56
100.0 %	15.7 %	20.4 %	16.6 %

Comparison between the values 53, 69 and 56 gives $P = 46$ %, (the same 0.1 cc. pipette used for withdrawal from the three tubes).

which forms part of a larger one, was made with a special dilution technique as far as the large doses are concerned, as it would often be quite impossible to count plates inoculated with $8 \times$ the normal dose, see also p. 88).

As to the number of leukocytes and bacteria a comparison between these—10-50,000 bacteria as compared to 5-10 mill. leukocytes—shows that even if the ratio may vary considerably there is always a massive majority of leukocytes (1 bact. pr. 100-1000 leukocytes). It appears from microscopical phagocytosis determinations that when the phagocytosis is powerful, a majority of the leukocytes takes part, each phagocytizing numerous bacteria (see for instance the following figures for the distribution of bacteria in 25 leukocytes after marked phagocytosis.—The figures were taken at random from Jersild's thesis (1942).

Example:	Number of bacteria per leukocyte:	40	20-40	10-20	1-10	).
	Leukocytes:	22	3	0	0	

The fact that most leukocytes are active when the phagocytosis is intense shows that in a culture experiment the same active leukocyte will only have few chances of phagocytizing more than one bacterium owing to the great

TABLE 9
*Bactericidal Experiment with Normal and eight Times Normal
Bacterial Density.*

In tubes with eight times the normal density the experiment was interrupted by transfer of 0.1 cc. to new tubes containing 7.9 cc. cold saline (dilution 1:80); in tubes with normal density the ordinary interruption by dilution 1:10 was used. Thus the difference in bacterial density between the tubes was eliminated before the inoculation, (cf. text on p. 88).

Serum conc. 55 %. Experimental culture: Breslau 206.

Density	Normal	8 x normal
Initial values	84	87
	91; $88 \times 2 = 176$	105; $96 \times 2 = 192$
Average:	184	
Values and degrees of bactericide:	54	46
	59	50
	66; 56	51; 49
	30.4 %	26.6 %

Comparison between the values 56 and 49 gives $t = 1.18$, computed from $s^2_{min.}$

preponderance of leukocytes referred to. The chances of a leukocyte being rendered inactive owing to overcrowding with bacteria will, of course, thus be minimal, and the consequence is that, with a given leukocyte density, the number of bacteria may vary rather freely without influencing the phagocytosis degree.

The said numerical relation between bacteria and leukocytes appears as a consequence of the fact that the new technique makes it possible to work with so weak bacterial suspensions. Besides the dilution of bacterial products aimed at, another advantage has been obtained which must be mentioned, even if its importance generally is not great: If we consider a phagocytosis experiment with a bacterial suspension containing groups of highly differently phagocytable elements, maximal or marked phagocytosis may easily be registered in microscopical examination, if only a sufficiently great supply of easily phagocytable bacteria is ready for each leukocyte. In contrast with this it is seen that by means of the culture technique a result will be obtained which is influenced independently of the number of bacteria, if for instance 25 % of the bacteria cannot be phagocytized. Moreover the culture technique permits an immediate examination of the question whether the bacteria that are free after the phagocytosis are in fact a selection of especially resistant elements.

Additional reagents: The additional reagents employed are citrate, oxalate, tartrate, anticomplementary serum, inactive serum, and immune sera. It applies to all these reagents that when stored in the right manner, they will keep well and the same portion can be used for long series of experiments; dilution and addition can thus be performed with the variations involved by pipetting and they are of slight importance—even to so comparatively sensitive a factor as citrate (see Chapter X). This does not mean, however, that as regards the said reagents comparisons may freely be drawn from one experiment to another. The single reagents influence either serum or bacteria, and as the latter two need not be open to influence to the same extent from one experiment to another, the same concentration of reagent need not always influence the experiment to the same degree (even if the influence tends in the same direction). Thus it applies in this case, too, that the free comparison is only permissible within the same experiment (in bactericidal experiments within such experiments as are performed with the same serum portion), whereas special control measures are necessary when passing from one experiment to another.

WITHDRAWAL AND MEASURING VARIATION

(2a and 2b)

The domains of variability 2a and 2b are in principle of the same content but of different importance to the estimation of the results of the experiments, 2a dealing with the variability of the single determination,

whereas 2b deals with the additional variability that must be expected when two single values are compared (when for instance the number of surviving bacteria has to be expressed in proportion to the initial value).

re 2a) By far the most important of the withdrawal and pipetting variations is the withdrawal variation of the relatively small number of bacteria transferred from the test tube to the agar plate. In order to arrive at a true estimate of its variance all counts of a longer experimental period have been included in the computations; only counts of the first few months' work with the new technique have been deliberately excluded.

Krag has shown that the withdrawal variation is highly dependent on two factors, the phase of growth at the time of inoculation and the composition of the milieu from which the withdrawal is made. The former of these factors is of no importance here, all the experiments being performed in the course of the lag phase of the bacteria; the latter factor might be imagined to play a part, though the differences in milieu that may occur after the dilution at the end of the experiment are slight in relation to those described by Krag.

In order to get an impression of the importance of the milieu, the material was first divided in the following groups:

- A 1. Bact. suspended in saline sol.—without centrifugation.
- A 2. " " " " " —with "
- B. Saline-phagocytosis determinations.
- C 1. Bact. suspended in serum milieu—without centrifugation.
- C 2. " " " " " —with "
- D. Serum-phagocytosis determinations.
- E 1. Bact. suspended in serum milieu + citrate.
- E 2. Phagocytosis determinations in serum milieu + citrate.

Within the single groups the material was divided in subgroups according to the mean colony figures (K). For each K the sum of the squared deviations from the mean (SK) was determined as well as the number of degrees of freedom (f = the number of single counts less 1). In each subgroup the mean value of all K ($\bar{K}$) and of all $V\{K\}$ ($V\{\bar{K}\}$)* was determined. The comparison between the various $V\{\bar{K}\}$ -values was then made by plotting $\bar{K}$ and $V\{\bar{K}\}$ against each other in coordinate systems in certain combinations of the groups A1-E2 (Table 10). In the figures lines are drawn corresponding to $V\{K\} = K$ and $V\{K\} = 1.2K$ and it is stated for each point on how many degrees of freedom the $V\{K\}$ in question is based. It applies to the 4 comparisons made that the points are freely distributed between or closely round the two lines. It may be concluded from this that there are no great differences in principle between the homogeneity of the bacterial suspensions in the different milieus.

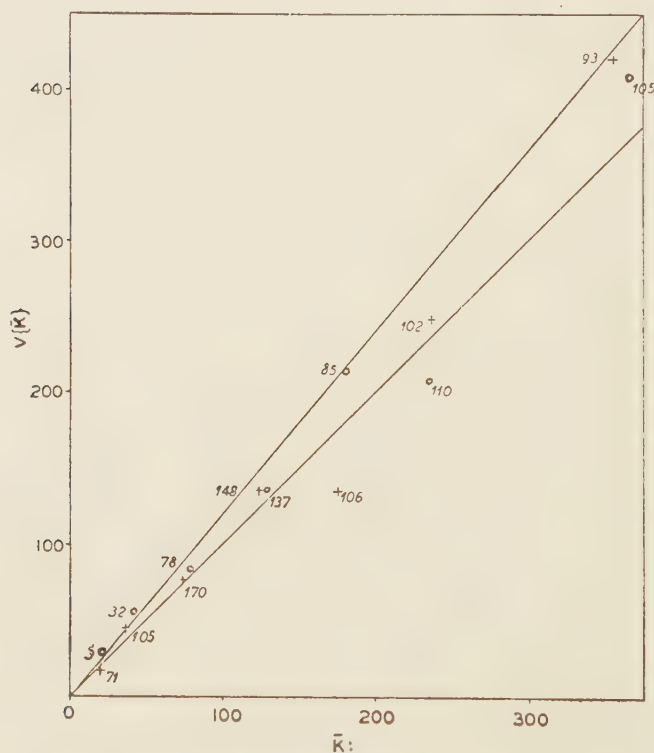
$$* V\{\bar{K}\} = \frac{\sum SK}{\sum f}$$

Lastly, on the basis of the homogeneity within the groups A1-E2, all determinations in each K-group have been collected, and in Table 11 the average values of all $\bar{K}$ and $V\{\bar{K}\}$ are seen plotted against each other. The resulting points, each based on 100-320 degrees of freedom very closely follow a line corresponding to $V\{K\} = 1.1 K$.

TABLE 10

. $\bar{K}$ - and $V\{\bar{K}\}$ -Values computed for Different Milieus plotted against each other.

The lines drawn up in the figures answers to $V\{\bar{K}\} = \bar{K}$ and $V\{\bar{K}\} = \bar{K} \times 1.2$. Next to each point is stated the corresponding number of degrees of freedom (f).

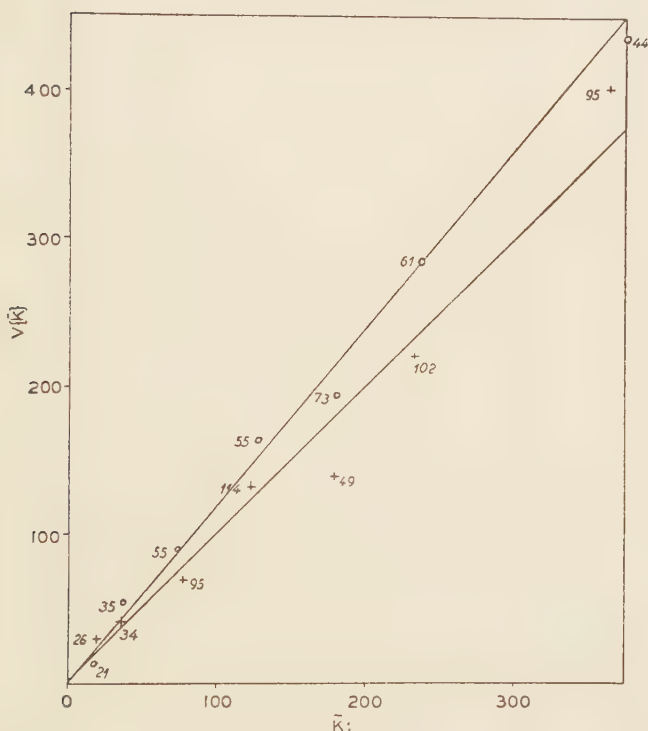


a) Figures computed from the groups A1 + A2 + B (experiments with bacteria in saline solution with or without blood corpuscles) marked with o.

Figures from the groups C1 + C2 + D (experiments with bacteria in serum milieu with or without blood corpuscles) marked with +.

In order more closely to examine the distribution of the $V\{K\}$ used for determining the different $V\{\bar{K}\}$ all duplicate determinations with $K < 10$ have been examined in one (i.e. the great majority of all determinations). The analysis of the material was made by means of the function $\chi^2 = \frac{SK}{s^2}$ (where s^2 is an estimate of the true variance σ^2) and, despite the association between $V\{K\}$ and K found graphically χ^2 was in the first instance determined as $\frac{SK}{K}$. The χ^2 -material (945 figures) was examined partly in different groups—4 chosen according to the composition of milieu and 5 according to the value of K —and partly in one (Table 13). The procedure

TABLE 10 (cont.)



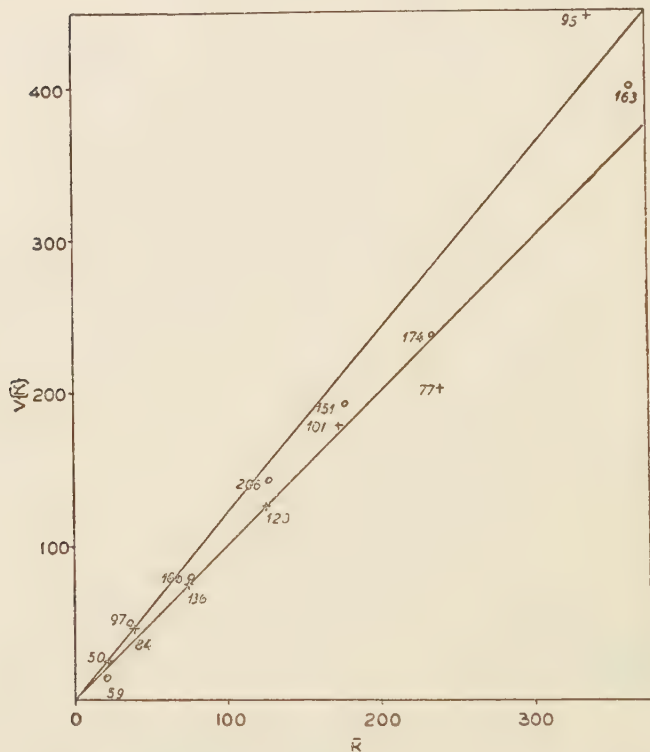
b) Figures computed from the groups A1 + C1 (experiments with bacteria in saline or serum, no centrifugation) marked with °.

Figures from the groups A2 + C2 (experiments with bacteria in saline or serum + centrifugation) marked with +.

followed was to divide the material of each group according to the value of χ^2 ; the limits for the different classes were chosen from Fischer & Yate's χ^2 -table, in such a manner that according to the theoretical distribution the values examined should be found to be distributed with 20 % (the lowest χ^2 -values) in the first class and 10-20-20-10-10-5-3-1 and 1 % respectively in the following classes. After this division of the χ^2 -material the number observed (o) in each class was compared with the expected number (v) and a new (secondary) χ^2 was determined according to the formula: $\chi^2 = \sum \frac{(o - v)^2}{v}$

The f-value corresponding to the sec. χ^2 equals the number of classes less 1, and on the basis of the χ^2 -table the probability percentages (P-values) cor-

TABLE 10 (cont.)



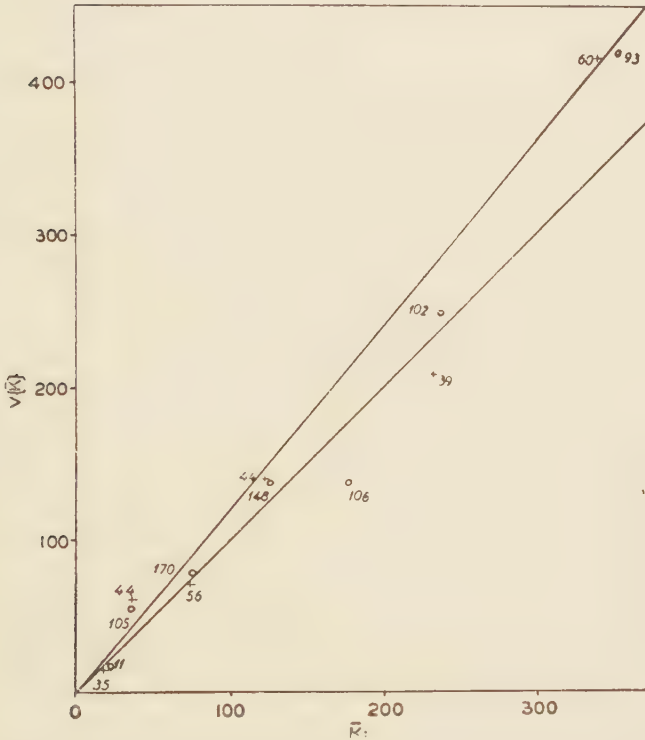
c) Figures computed from the groups A1 + A2 + C1 + C2 + E1 (all experiments without blood corpuscles) marked with o.

Figures from the groups B + D + E2 (all experiments with blood corpuscles in the tubes) marked with +.

responding to the sec. χ^2 -figures have been found from the χ^2 -table by interpolation. The method of calculation employed implies that neither observed nor expected value is less than 5 in any class and therefore it has been necessary in most cases to combine 2 or 3 of the smallest classes, for which reason the f -values are not the same in all groups in Table 13. It appeared that with the supposed standard deviation $s^2 = K$ the distribution was only acceptable in 3 out of the 4 and in 4 out of the 5 subgroups, and taken as a whole the χ^2 -material displayed a distribution highly deviating from the theoretical.

The distribution corresponding to $s^2 = 1.1 K$ and $s^2 = 1.2 K$ was

TABLE 10 (cont.)

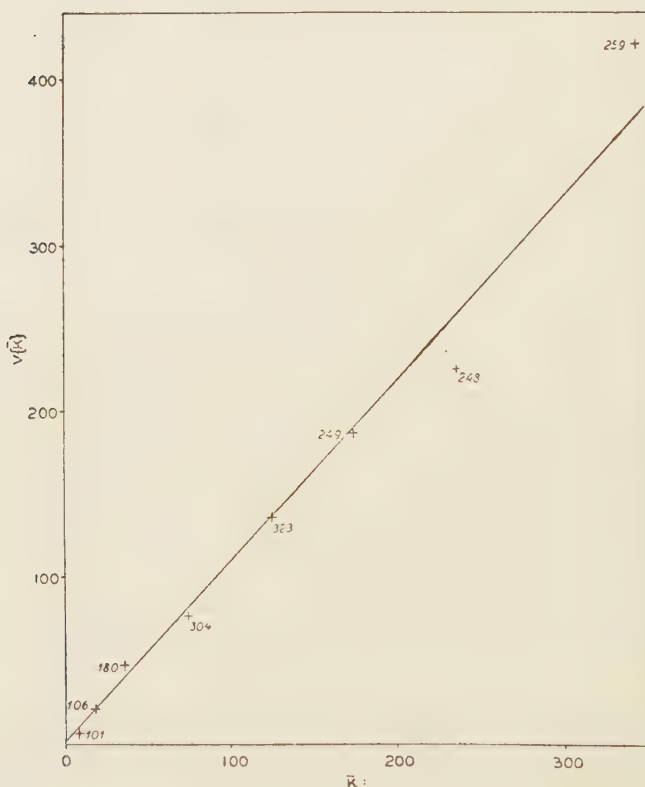


d) Figures computed from the groups C1 + C2 + D (experiments with bacteria in pure serum milieu with or without blood corpuscles) marked with o.

Figures from the groups E1 + E2 (experiments with bacteria in milieu with serum + citrate with or without blood corpuscles) marked with +.

therefore examined. The distributions found for both of the new s^2 -values in the total material and in all subgroups were acceptable except in the group C1 + C2, which still gave a highly deviating distribution. This proved to be due to an accumulation of χ^2 -values in the P-class 0.5 %; for which reason all χ^2 -values corresponding to $P < 5$ % were taken from the C-group for closer examination. The majority of the 37 values examined proved to originate from otherwise normal experiments and, therefore, had to be supposed to constitute the number of large χ^2 to be expected in a certain number of determinations; in two places, however, the accumulations were conspicuous: In 3 pure bactericidal experiments, per-

TABLE 11
 $\bar{K}$ - and $V\{\bar{K}\}$ -Values computed from all the Experiments in the Groups A1-E2 plotted against each other.



The line drawn up in the figure answers to $V\{\bar{K}\} = \bar{K} \times 1.1$. The numbers of degrees of freedom stated next to each point.

formed within the same week in Sept. 1944, 9 χ^2 -values* corresponding to $P < 5\%$ were found among 54 determinations and in one bactericidal experiment of Jan. 1944, 5 large χ^2 -values were found in 15 determinations. As will appear from Table 12, the accumulations of large χ^2 -values in the Jan.- and Sept.-experiments lead to frequencies that differ significantly from the frequency in the total material less the four striking experiments. Unfortunately it is impossible with certainty to decide the nature of the technical failures that have increased the number of large χ^2 -values in the experiments in question; it can only be pointed out that probably the fault is that a few test tubes in the said experiments have been subject to defective treatment after the dilution. If the mixing of the contents of a test tube is less thorough than usual—especially if the Pasteur pipette is not moved up and down in the test tube, or the pipette is so narrow that the amount of fluid passing it is much less than normal—then of course a less homogeneous bacterial suspension than usual will result. As to the Sept.-experiments it can be stated that for technical reasons the mixing had to be made especially rapidly; this may have involved a less careful treatment of some of the test tubes, and the fact that comparatively many large χ^2 -values are found, whereas the remaining values are of normal distribution, indeed seems to indicate that a few of the test tubes have been neglected. As far as the Jan.-experiment is concerned there are no special circumstances to report but the frequency of large χ^2 -values is so distinctly increased as to render the influence of abnormal conditions highly probable.

TABLE 12
Great χ^2 -Values in some Selected Groups.

	Jan. exp.	Sept. exp.	Jan. + Sept. exp.	All exp. — Jan. and Sept. exp.
Number of duplicate determinations	15	54	69	876
$s^2 = K:$	33.3 % $t = 2.7$	16.7 % $t = 2.3$	20.3 % $t = 3.3$	6.9 %
Number of χ^2 corresponding to $P < 5\%$ in per cent.	$s^2 = 1.1 K:$ 33.3 % $t = 2.9$	14.7 % $t = 2.2$	18.8 % $t = 3.2$	6.1 %
	$s^2 = 1.2 K:$ 33.3 % $t = 2.9$	14.7 % $t = 2.2$	18.8 % $t = 3.2$	6.1 %

The above t -values are drawn from a comparison between the percentage immediately above and the percentage in the same line of the last column.

* In reality 11, 2 of them, however, being doubtful owing to contamination of one plate in each double count.

On the supposition that owing to technical flaws the experiments referred to cannot be compared with the remaining material, new determinations of the χ^2 -distributions were made in the same groups as previously but after the Jan.- and Sept.-experiments had been excluded from the material. As 65 out of the 69 excluded determinations belong to the C-group and only 4 to the A-group, the correction almost exclusively concerned the distribution in the C-group and in the total material. The distribution of the C-group becomes acceptable for $s^2 = 1.1$ K and $s^2 = 1.2$ K, whereas the remaining subgroups and the total material form distributions which almost fit the theoretical distribution for $s^2 = 1.1$ K and the fit is nearly as good for $s^2 = 1.2$ K (see Table 13).

As referred to, the total variability found consists of the withdrawal + the pipette variation of the 0.1 cc. used for inoculation. To determine the latter a series of titrations were made; from an ordinary test tube filled with N/1 HCl, 0.1 cc. was taken with the usual technique, then being titrated with N/50 NaOH with phenolphthalein as indicator. On the basis

TABLE 13

P-Values corresponding to Secondary χ^2 's computed for Different Groups and for three Supposed Variances.

Milieu-groups	All duplicate determinations: P-val. calcul. for				Reduced material: P-val. calcul. for				f
	num- ber	$s^2 =$ K	$s^2 =$ 1.1 K	$s^2 =$ 1.2 K	num- ber	$s^2 =$ K	$s^2 =$ 1.1 K	$s^2 =$ 1.2 K	
A1 + A2 + B	178	90 %	98 %	78 %	174	90 %	98 %	68 %	7
C1 + C2	337	1 %	2 %	5 %	272	5 %	18 %	41 %	8
D	166	13 %	24 %	53 %	166	13 %	24 %	53 %	7
E1 + E2	264	7 %	25 %	31 %	264	7 %	25 %	31 %	8
K-groups									
11-40	183	1 %	11 %	34 %	178	1 %	8 %	13 %	7
41-80	147	34 %	59 %	58 %	133	7 %	42 %	56 %	7
81-140	194	23 %	31 %	32 %	179	55 %	54 %	70 %	7
141-240	220	52 %	82 %	90 %	200	61 %	89 %	90 %	7
> 240	201	25 %	46 %	31 %	186	19 %	35 %	19 %	7
Total	945	2 %	13 %	44 %	876	5 %	41 %	57 %	9

Milieu-groups: A1 + A2 + B = all determinations in saline milieu.

C1 + C2 = all bactericidal determinations in pure serum milieu.

D = all phagocytosis determinations in pure serum milieu.

E1 + E2 = all determinations in milieu with serum + citrate.

of 24 titrations the standard deviation was found to be 0.93 % of the volume withdrawn. From this it will be seen to how great an extent the withdrawal variation dominates; even in the case of large K-values, where the pipette variation plays the relatively greatest part, it is without practical importance. If we reckon with a standard deviation of 1 % in the pipetting and with a K of for instance 400, only 3.6 % of the total s^2 will originate from the pipette variation ($4^2 = 16$ out of $1.1 \times 400 = 440$); with lower K-values the pipette variation plays a still smaller part.

re 2b) As referred to, the additional variability with which we must reckon when comparing counts from two test tubes also consists of withdrawal and pipette variation, but as the bacterial suspension withdrawn is relatively dense, the mutual importance of the components differs from that previously stated.

Considering the fact that the mixing bottle is thoroughly shaken before bacteria are added to the test tubes, a greater homogeneity is doubtless obtained in the bottles than in the tubes where the contents are only mixed with a Pasteur pipette. The withdrawal variation must, therefore, have an $s^2 \leq 1.1 K$. The pipette variation of the 0.2 cc. added to each test tube was determined through titration as previously described, and on the basis of 24 titrations the standard deviation was determined to 0.84 % of the volume measured; there was no conspicuous difference between the sections of the 1 cc. pipette employed.

The importance of the variabilities referred to will best be seen from a numerical example: If for instance in the mixing bottle the bacterial density is 20,000 per 0.2 cc. the total s^2 will be $= 1.1 \times 20,000 + (0.84 \times 200)^2 = 50,224$, and the standard deviation will thus be about $224 = 1.14$ % of the number of bacteria withdrawn. This standard deviation will only vary slightly with the bacterial density, at 40,000 per 0.2 cc. it will thus be 0.99 %. In the following a standard deviation of 1 % of the number of bacteria withdrawn is reckoned with.

On comparison of counts from otherwise uniform test tubes still another pipette variability must, however, be reckoned with, owing to the fact that the 0.1 cc. pipettes employed are not completely uniform. Simultaneous with the determination of the measuring variation of 0.1 cc. a number of the pipettes used throughout a long period was examined. It appeared that the variation of the volume marked out is not inconsiderable; the standard deviation is about 2 % (determined through double titrations with 10 different pipettes). In the case of large colony figures this variability becomes of a certain importance, but it can be avoided if the same 0.1 cc. pipette is used for all withdrawals. This applies especially to experiments where it is desired to test identity or small differences, and the procedure was used in some of the experiments reported here (Tables 2, 8, 23, and 38); the pipette is rinsed between the withdrawal from two test tubes, the contents of the first pipette from the new tube being thrown away.

As will appear from sections 2a and 2b, we must reckon with the sum

of three—possibly only two sums of squared deviations from the mean when counts from the different test tubes are to be compared. The ultimate s^2 is found by adding the sums of squared deviations from the mean of the different scopes of variation and will thus be:

$$s^2 \text{ (total)} = \text{about } 1.1 K + (0.01K)^2 + (0.02K)^2;$$

(the last term is omitted if the same 0.1 cc. pipette is used in all withdrawals). The dominating 1st term of the expression for s^2 (total) is rather vaguely defined and, therefore, it would be most correct to state certain upper and lower limits for s^2 (total) and make use of them in the estimation of the experiments. If it is taken for granted that the true value of the 1st term lies between K and $1.2 K$, the former should be used in examinations for identity and the latter in demonstration of difference between counts from different test tubes. In this manner two s^2 (total) that can be used in practice are found:

$$\begin{aligned} s^2_{\text{max.}} &= 1.2 K + (0.01K)^2 + (0.02K)^2 \text{ and} \\ s^2_{\text{min.}} &= 1.0 K + (0.01K)^2 + (0.02K)^2 \end{aligned}$$

In Table 14 these quantities are drawn up as functions of K . The dotted curves state $s^2_{\text{max.}}$ and $s^2_{\text{min.}}$ for experiments where the same 0.1 cc. pipette was used for all withdrawals; as will appear from the Fig., only great K -values give an appreciable difference between the fully drawn and the dotted curves.

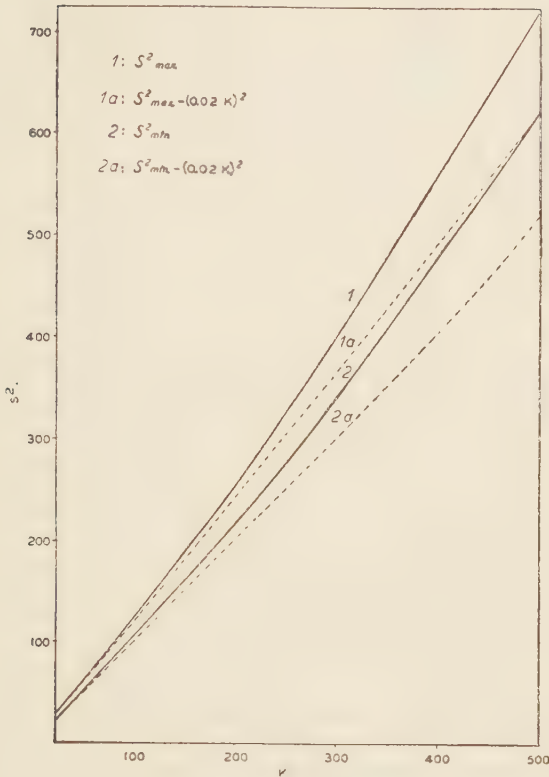
Experience from experiments where several test tubes would be expected to give the same result—e.g. surplus determinations at one end of a titration series—shows that the additional variability computed for comparison between counts from different test tubes hardly means any underestimate. Some instances are shown in Table 15: In 22 cases it has been possible to pick out several test tubes which in advance would be expected to give the same K ; from each series of tubes all single counts have been used for computation of common K and SK -values. $s^2_{\text{max.}} \& \text{min.}$ were computed from the common K -value, χ^2 was determined for both of them and the corresponding P -values are given in the Table. As will be seen, the computation on the basis of $s^2_{\text{max.}}$ gives preponderance of large P -values, which seems to indicate that $s^2_{\text{max.}}$ actually lies above σ^2 ; the P 's corresponding to $s^2_{\text{min.}}$ are almost theoretically distributed, but as the determination of $s^2_{\text{min.}}$ is based on a far greater material, there is hardly any reason to reduce the min.-value on the basis of Table 15.

Already on page 44 it was shown how the expressions for the total error computed above are used when different colony figures are compared. The following considerations of the experiment, Table 38, will illustrate the importance of the single factors to the computation:

In that experiment a comparison is made between the mean colony figures 469 and 494.5 determined from 7 and 15 single counts respectively; the fact that a significant difference can be determined between the two

TABLE 14

Graphic Representation of the computed $s^2_{max.}$ and $s^2_{min.}$



figures ($t = 2.30$) is, however, not solely due to the large amount of single determinations; it is of great importance that the colony figures are so large *and* that all withdrawals were made with the same pipette. Supposing a new pipette had been used for each test tube, $t = 2.12$ would have been found instead of $t = 2.30$; the considerable difference is due to the fact that the term $(0.02 K)^2$ of the expression for s^2 plays a comparatively great rôle in so large K-values. On the other hand, the fact that the large K-values are favourable under the given conditions is seen distinctly if the t-computation is made with halved K-values; such a computation gives $t = 1.65$ instead of $t = 2.30$, i.e. the significant difference has disappeared.

The uncertainty of t referred to on page 45 is very slight in this case; with $K > 400$ and $a + b = 22$ the computed t is within the limits $t \pm$ about 1 %.

TABLE 15

Survey of the Results from 22 Series of Test Tubes; in each Series the same K-Value expected from all Tubes.

K	Number of tubes	f	P computed for	
			$s^2_{\text{max.}}$	$s^2_{\text{min.}}$
40	3	8	2.5 %	1.2 %
301	3	8	8 %	5 %
418*	5	13	16 %	9 %
223	3	8	26 %	16 %
106	4	8	31 %	18 %
271	4	10	38 %	20 %
62	3	6	41 %	31 %
345	3	6	48 %	37 %
344	4	8	49 %	37 %
59*	3	8	52 %	38 %
168	2	7	60 %	49 %
332	4	11	62 %	49 %
495*	5	14	64 %	51 %
316	7	13	65 %	50 %
136	5	9	71 %	54 %
30	5	14	80 %	65 %
86	3	6	81 %	76 %
124	3	8	90.95 %	88 %
154	4	15	90.95 %	88 %
228	3	8	90.95 %	89 %
538	3	8	90.95 %	90 %
20	6	11	95.98 %	90.95 %

With * are marked values from experiments in each of which the same 0.1 cc. pipette was used for withdrawal from all tubes, (i.e. Tables 23, 8 and 38). In these cases, on computing the s^2 -values, the last term in the expressions for $s^2_{\text{max. and min.}}$ has accordingly been omitted.

PART TWO

CHAPTER IV

EXPERIMENTS WITH HEATED NORMAL SERUM

Hitherto heated inactive serum has as a rule been considered an entirely neutral medium for phagocytosis. Wright & Douglas (1904) thus showed that the phagocytosis degree decreased in the same manner whether the active serum was diluted in saline solution or in heated serum. Later on Bächer (1907) confirmed that inactive serum does not influence the phagocytosis process. The experiments on which the supposition of the indifference of inactive serum is based were, however, performed with a technique that does not allow the serum concentration to exceed about 33 per cent.; the experiments were moreover made with dense bacterial suspensions, the sensitivity of the phagocytosis system to slight alterations of activity thus not being very great.

The technique employed in the writer's investigations, allowing a serum concentration of up to 80 per cent., showed that it is not correct to consider inactive serum an indifferent factor in all cases.—The importance of heated serum is especially conspicuous when the phagocytosis degrees in saline milieu are compared with those in a milieu with a high concentration of heated serum. The first assumption was that the *inhibition* of the phagocytosis process seen on addition of inactive serum might be due to merely physical conditions: If for instance an increase of the viscosity of the fluid phase influenced the motility in the phagocytosis system so as to reduce considerably the chances of interference between bacteria and leukocytes, an addition of inactive serum would inhibit the phagocytosis solely for that reason. In order to examine these conditions more closely some experiments were performed with examination of the phagocytosis degrees in milieus of varying viscosity; comparisons

were made between saline-phagocytosis and phagocytosis in milieus with high concentrations of heated serum, or with artificially increased viscosity. The experiment (Table 16) distinctly shows *both* the characteristic inhibition of the phagocytosis produced by inactive serum *and* that it cannot be due to increased viscosity. For the purpose of increasing the viscosity artificially cellulose was used, which has the advantage of being chemically highly indifferent but proved to have a peculiar secondary effect, as it increased the sedimentation rate of the erythrocytes

TABLE 16

Spontaneous Phagocytosis in Milieus with Different Viscosity.

The relative viscosity determined by the time required for a given volume to empty itself through a pointed Pasteur-pipette.

I: Saline solution;		time of outflow:	25.7 sec.
			25.8 "
			26.0 "
II: 55 % heated serum (55°, 30');		" " "	34.8 "
			: 35.1 "
			35.0 "
III: Saline solution + 7 vol.% of cellulose;		" " "	35.8 "
			: 35.2 "
			36.1 "

Experimental culture	Breslau 1	Breslau 206	Breslau 206*
Initial values	243	122	
	228	128	121
	242; 238 × 2	141; 130 × 2	145; 133 × 2
	476	260	266
Values and degrees of phagocytosis:	286	75	
	290	83	
	342; 306	85; 81	
	I 64.3 %	31.4 %	
II	473		
	478	237	
	485; 479	249; 243	
	100.6 %	93.5 %	
III	252	88	
	276	92	
	280; 270	103; 94	
	56.8 %	36.4 %	

* Control determination with bacteria in saline + cellulose.

very much; in the course of the experiment the test tubes thus had to be shaken several times to keep the blood corpuscles suspended.

A few comments must be made on the sedimentation rate: The different mixtures that have been used for re-suspension of washed blood corpuscles can be arranged in the following manner according to increasing sedimentation rates: Saline solution, fresh serum, serum heated to 56° , serum heated to 60° , and lastly cellulose-saline. In the case of the mixtures the sedimentation rate increases with the concentration of serum or cellulose. Only when high concentrations of serum heated to 60° , or cellulose were used, shaking of the test tubes more than once in the course of the experiment was required.

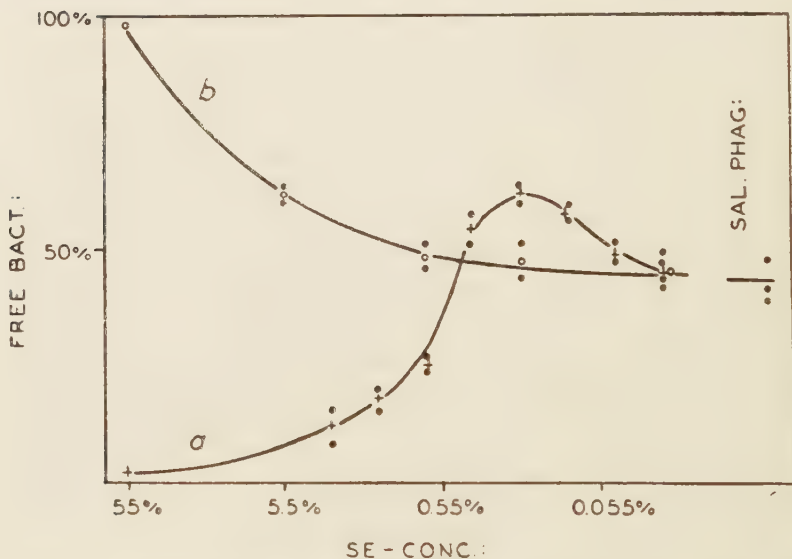
The difference between the influence of the fresh (active) serum and that of heated serum on the phagocytosis will appear very distinctly from the experiment reproduced in Table 17. Two series of phagocytosis determinations (two titrations) were made in that experiment; in one series—curve a—the concentration of active serum falls from 55 per cent. to about 0.02 per cent., whilst the second series shows determinations with corresponding concentrations of inactive serum—curve b. What is most conspicuous is the diametrical opposition between the effects of high concentration of active and inactive serum respectively, but the experiment also reveals another peculiar phenomenon: The lowest concentrations of active serum exercise a paradoxical effect—they *reduce* the phagocytosis. In other words, the lowest degrees of opsonin influence reduce the phagocytability of the bacteria. The course of curve b shows that the paradoxical effect is not—as might be imagined—due to simple summation of the two reverse phagocytosis influences of active and inactive serum respectively.

As is shown by the single determinations represented in the figure, the paradoxical effect of active serum is a real one; it is, however, so slight as to manifest itself distinctly only when the experiment is performed with a bacterial strain whose saline-phagocytosis value (= the limit of the titration) is near 50 per cent. If the colony figures proper of the experiment in Table 17

are evaluated against each other, the two highest values of the paradoxical inhibitory phase (4 determinations) being compared as a whole to the values characterizing the final level of the titration (6 determinations), the result will be a highly significant difference with a $t = 3.60$.

For the time being it is impossible to give any reasonable explanation of the paradoxical effect, and otherwise the phenomenon has been of no practical importance. Owing to the inhibitory influence which inactive serum proved to exercise on the phagocytosis care was taken in all subsequent titration ex-

TABLE 17
Titration of the Effect of Active and Inactive Serum respectively on Phagocytosis.



Maximal serum conc. 55 %, both active and inactive serum diluted with saline solution.

Curve a: Active serum, mean values marked with +.

„ b: Inactive serum (heated to 55° for 30 min.), mean values marked with o. Single determinations marked with dots.

Initial value: 193. Degree of saline-phagocytosis (sal. phag.): 43.0 %.

Experimental culture: Breslau 50.

periments to keep the total concentration of serum (active + inactive) at a constant level (about 50 per cent.). This implies that when the opsonin activity decreases, such titration curves do not approach the saline-phagocytosis value but the value of phagocytosis at a high concentration of inactive serum, i.e. values of 70 to 90 per cent. free bacteria. This again means that almost the entire course of the titration can be observed without being disturbed by possible inhibitory phenomena like the one seen in Table 17 and, as already referred to, the inhibitory phenomenon is moreover so slight that it will hardly manifest itself if the limit of the titration is at so high a level as for instance 80 per cent. free bacteria.

In the case of the bactericidal process, too, it can be shown that inactive serum is no indifferent milieu factor. In contradistinction to the phagocytosis experiments it is, however, necessary always to have a certain portion of active serum in the test tubes as, in a bactericidal experiment, there is no equivalent for the saline-phagocytosis value that can be used as a starting point for determination of a possible inhibitory effect. If it is desired to demonstrate such an effect it will therefore, be necessary to perform the experiment with a bacterial strain that is highly sensitive to alexin so that with a small dosis of active serum a suitably low (basal) percentage of survival can be arrived at. From this basal value the possible inhibitory effect of inactive serum can then be measured.

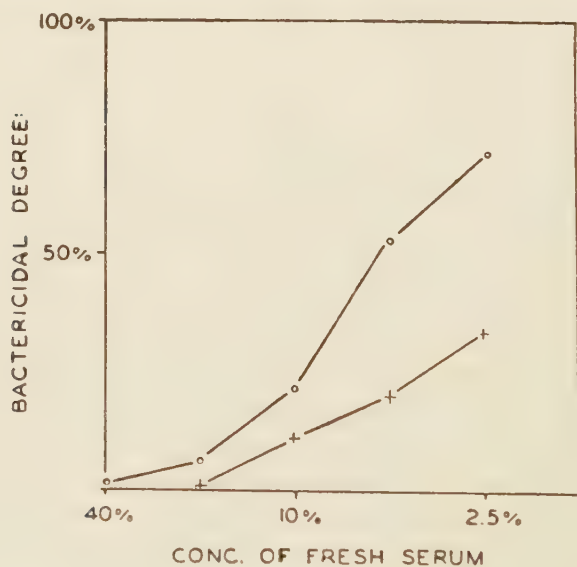
By means of such a sensitive bacterial strain it can easily be shown that a sufficiently large addition of heated serum exercises an *inhibitory effect* on the bactericidal process. The experiment in Table 18 represents two series of bactericide determinations with equally falling concentrations of active serum; in one series the active serum is diluted with saline solution, in the other with heated serum in such a manner that the total concentration of serum is kept at a constant level of 80 per cent. In graphic representation the experiment displays curves that are distinctly displaced in relation to each other; the inhibitory effect of the inactive serum is seen most distinctly in the "sensitive" range, i.e. when corresponding values of the two series are

situated fairly symmetrically about the 50 per cent.-line, as is the case at a concentration of fresh serum of 2.5 per cent. The experiment moreover shows that a very large addition of inactive serum is required to produce distinct inhibition.

The interdependence between the total serum concentration and the degree of inhibition was examined in an experiment in which three different concentrations of active serum were used as basal doses to determine the degrees of bactericide at increasing concentrations of inactive serum (Table 19). The experiment shows that it is the total serum concentration that determines when inhibition begins; it thus applies to all the three concentrations of active serum that only at a total concentration of more than 40 per cent. do safe inhibitory effects appear.

TABLE 18

Bactericidal Experiment with and without Addition of Heated Serum.



Curve marked with +: Active serum diluted with saline.

" " " 0: " " " " heated serum, total serum conc. constant = 80 %.

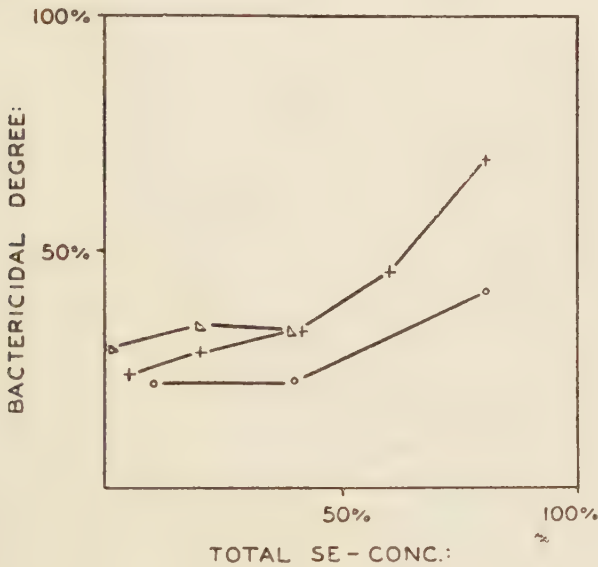
Initial value: 300. Experimental culture: Breslau 206.

If, for instance, instead of the total concentration it were the ratio between the concentrations of active and inactive serum that determined the occurrence of inhibition, a distinct inhibition would have occurred with the combination of 2.5 per cent. active serum and a total concentration of 40 per cent. (ratio 1:16), judged by the intermediate curve in Table 19, whereas with 10 per cent. active serum practically no inhibition would have occurred, even with a total concentration of 80 per cent. (ratio 1:8).

As to the substances in heated serum exercising an inhibitory effect on phagocytosis and bactericide it is important, with a view to subsequent determinations, to decide whether these substances are found in genuine serum or whether they are formed

TABLE 19

Bactericidal Experiment with and without Addition of Heated Serum.



Curve marked with Δ : Basal dose of active serum 2.5 %.

" " " + : " " " " " 5.0 %.

" " " o : " " " " " 10.0 %.

Initial value: 210. Experimental culture: Breslau 206.

in serum in the course of heating. In the case of phagocytosis inhibition the question is readily answered: A number of titration experiments that will be dealt with more closely in the following have shown that if, in a milieu with for instance 50 per cent. active serum, the serum activity is greatly inhibited (e.g. by adding a small amount of anticomplementary serum) a phagocytosis degree is obtained that is identical with that of a control test tube containing 50 per cent. inactive serum. If such an experiment is performed with a strain that is readily phagocytable in saline milieu (e.g. Breslau 206) the saline-phagocytosis degree (about 30 per cent. free bacteria) and the identical phagocytosis degrees in the tubes with heated serum and with active + anticomplementary serum (about 80 per cent. free bacteria) are so clearly different that the following conclusion may be drawn: *Heated serum and fresh serum, the activity of which is greatly inhibited without heating of the serum, will inhibit the saline-phagocytosis alike. This seems to indicate decisively that the inhibition is brought about by substances contained in genuine serum.*

It can be added that the same relations are found if the activity of the fresh serum is inhibited by means of NH_3 -treatment a.m. Gordon; see Table 20, first column at the bottom.

In the case of inhibition of the bactericide it is more difficult to decide whether the inhibition is due to genuine serum substances or abnormal products formed in the course of heating. It is an old-established fact that an addition of heated serum reduces the hemolysis degree of a hemolysis system that is balanced beforehand. Morrison (1922) pointed out that the inhibition might be due to adsorption of complement to abnormally large protein molecules of the heated serum. Although a mechanism of that kind cannot be of any great importance to the inhibition of phagocytosis, which is just most marked in alexin- and opsonin-free milieu, it might well be imagined beforehand that alexin adsorption was the cause of the inhibitions of bactericide described above.

As an attempt to settle the question whether the inhibitions of phagocytosis and bactericide produced by inactive serum are

released by the same or different agents, experiments of two different types were performed:

1) Inhibition experiments with sera inactivated at different temperatures and for varying periods.

2) Experiments with preliminary treatment.

re 1) The sera employed were treated for 15 to 120 min. at temperatures of 55° , 60° or 63° . 3 experiments with addition of sera treated in this manner are embodied in Table 20, in which the vertical columns contain either a bactericidal or a phagocytosis experiment; at the top of the table saline-phagocytosis values and the basal bactericidal values are stated; at the bottom the nature of the experiment and its total serum concentration are stated.

Table 20 shows that, even if the inhibition of the phagocytosis must be supposed to be due to substances contained in genuine serum, the inactivation conditions are not without importance to the degree of inhibition obtained. The proportion between the inactivation temperature and time and degree of inhibition, however, changed from one experiment to the other. In a couple of cases the inhibition was found to increase with the inactivation temperature but to be independent of the duration; in a subsequent experiment, however, increasing inhibition was found with an extension of the inactivation period, but slighter inhibition at 60° than at 55° . The reason of the varying results is unknown and the question will not be discussed in detail; the most essential feature of the experiments is at present that within the same experiment the inhibitions of phagocytosis and bactericide have constantly shown a common dependence on temperature and duration of inactivation.

re 2) As normal serum substances can hardly be imagined to produce any injury to leukocytes, thus decreasing the phagocytosis, it must presumably be through some influence on the bacteria that the inhibition of phagocytosis of the heated serum is brought about. It should thus be possible to obtain valuable information by means of experiments with preliminary treatment.

In these experiments the bacteria were submitted to about 10 minutes' preliminary treatment at a high concentration of heated serum and then transferred to test tubes with washed

TABLE 20

Instances of Bactericidal and Phagocytosis Inhibition produced by Addition of Normal Sera inactivated in Different Ways.

Experimental cultures	Breslau 206A	Breslau 206A	Breslau 206	Breslau 206
Initial values:	169	about 240	about 310	
Bactericidal values (5 % active serum):		43 57; 50	29 33 27; 30	
Values of saline phagocytosis:	29 38 39 40; 37	73 80; 77	123 137 142; 134	
Experimental values after addition of serum inactivated by the following treatments	102 103 138; 114		262 267; 265	149 154; 152
	55°, 15'			
	97 100; 99	183 212 231; 209	100 104 118; 107	260 274 283; 272
	55°, 30'			167 176 179; 174
	106 107; 107			194 227; 211
	55°, 60'			
	98 104; 101			
	55°, 120'			
	113 130; 122	228 243 251; 241	128 144 156; 142	245 268; 257
	60°, 30'			115 137; 126
	129 134; 132			258 282; 270
	60°, 60'			149 171; 160
	128 130; 129			287 304; 296
	60°, 120'			
	138 145; 142			
	62°-63°, 30'			
	116 145; 131			
	NH ₃ *			
Added serum in per cent:	27.5 %	45 %	55 %	55 % 75 %
Nature of experiment	Phagocytosis	Phagocytosis	Bactericide	Phagocytosis Bactericide

The average of the mean values 114, 99, 106 and 101 in the first column is: 106; the average of the values 122, 132 and 129 in the same column is: 127. Corresponding to these averages we find $P = 27\%$ and $P = 87\%$ respectively, (cont. at bottom of next page).

* For details concerning the NH₃-treatment see chapter VII, Table 28.

blood corpuscles or a small portion of active serum. For the purpose of comparison control tubes were used to which serum of the same concentration as the one obtained in the former tubes by transferring bacteria + serum from the milieu of preliminary treatment had been added. In order to be sure that experimental and control tubes received the same amount of bacteria the addition to all test tubes took place by means of the same dilution procedure, i.e. from a stock suspension of bacteria identical volumes were added to two "preliminary treatment tubes"—one with heated serum and one with saline solution. 10 minutes afterwards equal volumes were then transferred from these tubes to experimental test tubes and control tubes respectively. Table 21 represents two experiments with preliminary treatment performed with this technique.

The experiments with preliminary treatment, too, gave somewhat varying results. As a rule the bacteria submitted to preliminary treatment displayed slightly decreased phagocytability *and* slightly decreased sensitivity to alexin; still, in one case the preliminary treatment was without any effect whatever. Through preliminary treatment in heated normal serum anything like the degree of inhibition of phagocytosis or bactericide described above was never successfully transferred. Thus the experiments certainly seem to indicate that the bacteria are influenced in the course of the preliminary treatment but also that this influence is highly reversible. In trying to find an explanation of the phenomena observed it must be imagined that at a high concentration of inactive serum certain substances are bound to the bacteria, their phagocytability and sensitivity to alexin thus being reduced, but that this binding is a loose one and that the substances bound are therefore again split off when the serum concentration is reduced, as will happen when the bacteria are transferred from the milieu of preliminary treatment to the experimental test tubes.

TABEL 20 (cont.)

Comparison of the above values 106 and 127 gives $t = 3.29$.

" " " values 209 and 241 in the second column gives $t = 2.29$.

" " " " 107 " 142 " " third " " $t = 3.40$.

All t -values calculated from $s^2_{\max}$.

The experiments described with sera inactivated in different ways and the experiments with preliminary treatment thus did not disclose any difference between the normal substances inhibiting phagocytosis and bactericide. On the basis of the experiments hitherto reported any identification of the two substances cannot be ventured, however; we must be content to ascertain a certain resemblance between them.

The experience about the inhibitory effects of the inactive normal serum can now be summarized as follows:

There is a reason to believe that the inhibition of phagocytosis is neither due to injury to the leukocytes nor to altered

TABLE 21

Bactericidal and Phagocytosis Experiments with Bacteria Preliminarily treated with Inactive, Normal Serum in High Concentration.

The inactive serum was heated to 55° for 30'. The duration of the preliminary treatment was 10-15 min.

Experimental cultures	Breslau 1		Breslau 206
Initial values	166		about 300
Values of saline-phagocytosis:	80 103 113; 99		
		Bactericidal values in 5 % active serum:	16 23 24; 21
Values of phagocytosis in 5.5 % inactive serum:	101 110; 106*	Bactericidal values in 5 % active + 35 % in- active serum:	26 29 47; 34**
The same, only bacteria treated first in 55 % in- active serum:	131 137; 134*	The same, only bacteria treated first in 75 % in- active serum:	38 50 53; 47**
Values of phagocytosis in 55 % inactive serum:	163 164; 164	Bactericidal values in 5 % active + 75 % in- active serum:	120 156; 138
Nature of experiments:	Phago- cytosis		Bacteri- cide

Comparison between the values * 106 and 134 and the values ** 34 and 47 gives $t = 2.26$ and $t = 2.27$ respectively as calculated from $s^2_{\max}$.

motility in the phagocytosis system. According to the importance that may be ascribed to a possible alexin adsorption (Morrison) there will then be two possibilities of describing the inhibition mechanism:

1) The inhibition of the phagocytosis and bactericidal effects may be imagined to be produced by different routes: When, at a high concentration of inactive serum, the bacteria are influenced so as to make their binding to the leukocytes more difficult it might theoretically take place without any change of the possibilities of action of the opsonin. In that case, in a phagocytosis system with fresh + inactive serum, there would be two phagocytosis influences acting reversely and independently—i.e. the promoting effect of the opsonin and the inhibitory effect of the thermostable serum substance.

Besides such inhibition of the phagocytosis independent of the fresh serum, opsonin and alexin adsorption might be imagined as concurrent cause of the inhibition of phagocytosis in a milieu containing opsonin and as the sole cause of the inhibition of bactericide.

2) The inhibition of the phagocytosis and bactericidal effects may be imagined to be produced by the same agent: If so, that agent must be imagined to possess a double effect, influencing the bacteria so that they become *both* less phagocytable in saline milieu *and* less sensitive to alexin.

The experiments reported hitherto will hardly allow the choice of either of these two possibilities. As referred to, only the demonstration of a certain resemblance between the normal substances inhibiting phagocytosis and bactericide has been successful.

CHAPTER V

IMMUNE SERUM EXPERIMENTS

The inhibition phenomena referred to in the preceding chapter will—at any rate in the case of inhibition of phagocytosis—occur when bacteria are influenced by a normal thermostable serum component in high concentration. If it is supposed that the inhibitory factor is identical with the “normal antibody” referred to in the introduction, the inhibition phenomena will bear a considerable resemblance to the well-known Neisser-Wechsberg phenomenon which, as is known, is released by specific antibody when the latter is present in relatively high concentration and which is observed in many different connections (phagocytosis, bactericidal, agglutination and precipitation experiments).

It is briefly referred to in the introduction that in cases where a phagocytosis, bactericidal or hemolysis process takes place without the presence of specific antibody, the place of the latter in the system is taken by the “normal antibody”. Among the many investigators who have demonstrated the presence of such a normal factor the following can be mentioned: Moxter (1899), Levaditi (1901), Sachs (1902), Simon, Lamar & Bispham (1906), Muir & Browning (1906 and 1909), Cowie & Chapin (1907), Hata (1903), Gordon and collaborators (1928 and 1932) and Mackie & Finkelstein (1930 and 1931).

There always has been and still is some uncertainty about the specificity of the normal antibodies. Muir & Browning found that absorption of the normal factor was *often* most easily recognizable when the same bacterial strain as had been used for the absorption was used to determine the decline of the alexin activity of the absorbed serum. This might seem to indicate that

the removed normal antibody is specific to some extent, provided the relation between the alexin-sensitivity of the different bacteria is sufficiently considered. Mackie & Finkelstein went much further; they advanced the supposition that normal serum contains a complete spectrum of specific antibodies; a point of view the consequence of which is that the immune bodies observed in *large* quantities after immunization only constitute "specific" increases of the concentration of pre-existing and actually normal serum substances.

Most investigators—both before and after Mackie & Finkelstein's publications—have, however, arrived at the result that the normal antibodies are but slightly specific. Dunlop (1928) thus stated that by means of treatment with active coal or glass powder guinea-pig serum could be deprived of a normal factor which, together with bacteria, produces inhibition of hemolysis (zone phenomenon). Gordon & Carter (1932) stated that with a given absorption culture they were able to reduce or discontinue the alexin action against a number of different bacteria and that the effect on the least sensitive strains subsided first; (these experiments were performed with so small absorption doses that the complement content of the serum treated was practically normal; as referred to before, other investigators have generally used absorption at a low temperature to avoid a decrease of the complement content in the course of absorption).

From a quantitative viewpoint, too, the idea of one or at any rate few and fairly unspecific "normal antibodies" appears to be most reasonable. It is rather difficult to imagine that serum should contain a sufficient amount of antibody to sensitize a greater number of bacteria of a culture chosen at random if the countless type antigens, whose existence is revealed in the course of immunization experiments, were represented in normal serum each by its own specific antibody.

At first glance it might seem most natural to examine the importance of the normal antibody to the inhibition phenomena referred to by trying to remove the inhibitory properties of inactive serum through absorption with bacteria. It was, however, not ventured to use this procedure owing to the aggressin-like effect that may be observed in culture filtrates and in absorbed

and filtered serum. This effect can be demonstrated by adding to a phagocytosis or bactericide test tube a non-bacterial filtrate of culture, or serum in which large amounts of bacteria have been suspended; such an addition reduces both the alexin and opsonin activity. Table 22 gives an instance of the effect of absorption of a normal serum with a bacterial culture chosen at random (typhus H 901). As will be seen, almost all bactericidal and phagocytosis effect is discontinued after the absorption, but at the same time the absorbed serum has in itself become inhibitory to phagocytosis to a fairly great extent, which can be seen from the control test tube to which, besides fresh serum, absorbed serum has been added. Other experiments have shown that the inhibition exercised by such an absorbed and filtered serum or by a non-bacterial filtrate of bouillon culture comprises both the bactericidal and the phagocytosis processes and is not due to injury to the leukocytes in the cases examined.

It is possible that the inhibitory effect just occurs because filtrable bacterial products take possession of some of the normal antibody, but the possibility cannot be ignored that the aggrasin-like products may have a more direct anti-alexin effect. The

TABLE 22

Bactericidal and Phagocytosis Experiment with Serum treated with Absorption.

Absorption material: Live culture of typhus 901 H, (the culture somewhat contaminated).

Serum a: 10 cc. fresh serum + the centrifugate from 20 cc. of a dense suspension of typhus bacteria incubated at 37° for 45', centrifuged and the serum passed through a glass filter (Schott & Gen., Jena: 3G 5 auf 3).

Serum b: Fresh control serum, centrifuged and filtered as serum a.

Experimental culture: Breslau 1.

Experimental milieu	Saline	Serum a 50 %	Serum b 50 %	Serum b 50 % + serum a 25 %
Values and degrees of bactericide:	320 329; 325 100.0 %	299 324; 313 96.6 %	243 248; 245 76.2 %	
Values and degrees of phagocytosis:	268 269; 269 83.3 %	253 256; 255 79.0 %	16 22; 19 5.9 %	99 100; 100 31.0 %

experiments hitherto performed by the author with culture filtrates and absorbed serum gave no safe information about the mechanism of the influence of the said products on the alexin and opsonin processes, and as long as this mechanism remains unknown it is impossible to use absorption as a test to show that some *certain* component of the alexin-opsonin-system—in this case the normal antibody—has been removed.

It has, however, been possible by other methods to arrive at a supposition of the importance of the normal antibodies to the inhibition phenomena described in the preceding chapter. It has been mentioned that if the inhibition by normal serum of the saline-phagocytosis and the bactericidal effect is exercised by one agent, it must be ascribed to a double effect, namely that through the influence of this agent the bacteria become less phagocytatable in saline milieu and at the same time less sensitive to alexin and opsonin. In the following it is intended to demonstrate that such a twofold effect is produced by specific antibody in a suitably high concentration.

It might be expected in advance that the special technique employed in the present work would not allow the performance of experiments with addition of immune serum, which might be expected to produce agglutination, thus rendering reliable counts impossible. Experience from numerous experiments of different arrangement showed, however, that no agglutination occurs in the weak bacterial suspensions employed and within the comparatively short duration of the experiments.

For all the experiments referred to in the following the writer used immune sera inactivated through heating to 56° for 30 min. Table 23 represents a simple agglutination control performed with a highly agglutinating rabbit serum in different concentrations. The constant numbers of bacteria from all the test tubes show that no agglutination occurred.—As to the stability of weak bacterial suspensions in immune sera, see also the experiment, Table 25.

In the following some typical experiments with addition of immune serum are reported:

It is known that the binding between bacteria and immune body is very rapidly established. Bjørneboe (1940) for instance

showed that under suitable conditions there is no difference between the amount of agglutinin that is bound in the course of 5 minutes and the amount that can be bound in the course of 48 hours. To make sure that the immune serum experiments give a clean picture of the fate of bacteria that have been sensitized by specific antibody, it was decided to begin the experiments by mixing bacterial suspension and immune serum dilution (as a rule 0.2 and 0.3 cc. respectively). When these volumes have been pipetted off, the rack is shaken and left at room temperature for 5 to 10 minutes; when, in the course of this preliminary treatment, the bacteria have had an opportunity to bind immune body the actual experiment is started, active serum and (or) blood corpuscle suspension being added and the rack shaken again. In the following experiments the concentrations of immune serum stated refer to the actual experimental milieu, i.e. the concentration of immune serum in the preliminary period has been twice the figure stated.

Several former investigators (among others Thjötta, 1938) showed that, according to the concentration, immune body will promote or inhibit the bactericidal process; both of these phases of the effect will appear distinctly from the experiments in Tables 25 and 26. As to the inhibitory phase it should be especial-

TABLE 23
Control for Agglutination.

The bacterial density determined in saline solution and in saline solution + different amounts of heated rabbit immune serum. The serum (S1) is pooled serum from three rabbits immunized with live Breslau 1; its agglutination H-titer is about: 1:10,000.

Experimental culture: Breslau 1.

Pure saline solution	Saline solution + S1 diluted,			
	1:40	1:160	1:640	1:2560
	392	408	390	401
392	398	434	430	408
403; 398	447; 413	478; 440	454; 424	415; 408

Comparison of all 5 mean values gives $P = 11\%$, (the same 0.1 cc. pipette used for all withdrawals).

ly emphasized that with increasing concentration of immune body does not only its promoting effect cease, the inhibition also interferes with the basal effect produced by the active serum, which is discontinued wholly or in part. *The inhibitory effect of the specific antibody thus manifests itself distinctly in the range where the inhibitory effects of the normal, heated serum is observed.*

TABLE 24

Partial and Total Inhibition of the Bactericidal Effect resulting from Addition of Specific Rabbit Immune Serum in High Concentration.

Specification of the immune sera employed:

S1—Immunization with live Breslau 1 (see Table 23).

S2— " " " " 1 (produced later than S1).

S3— " " Breslau 1 killed by formalization (H + O-serum).

S4— " " heat-killed Breslau 1 (O-serum).

S5— " " live salmonella Thompson, (this serum has no agglutinin in common with Breslau-sera and is used as control).

Experimental cultures		Breslau 206	Breslau 206	Breslau O-form
Initial values		333		
		335	171	116
		360; 343*	180; 175.5 × 2	123; 119.5 × 2
			351	239
	saline (basal values)	63 64; 64	31 38; 35	26 38; 32
Bactericidal values in active serum + 55 % of the following reagents:	S1 1:100	338 350; 344*		
	S2 1:100	300 365; 333*		
	S3 1:100	343 368; 356*		
	S4		81 83; 82**	198 212; 205***
	S5 1:100	60 71; 66		

** S4: 1:400, *** S4 1:20.

Comparison of the mean values * 343, 344, 333 and 356 gives $P = 44\%$.

Table 24 gives some instances of total and partial blockade of the basal effect brought about by means of different specific immune sera. The control experiment with salmonella Thompson-serum seems to indicate that the inhibitions observed are inherent of sera containing agglutinin against the bacterial strain employed in the experiment.

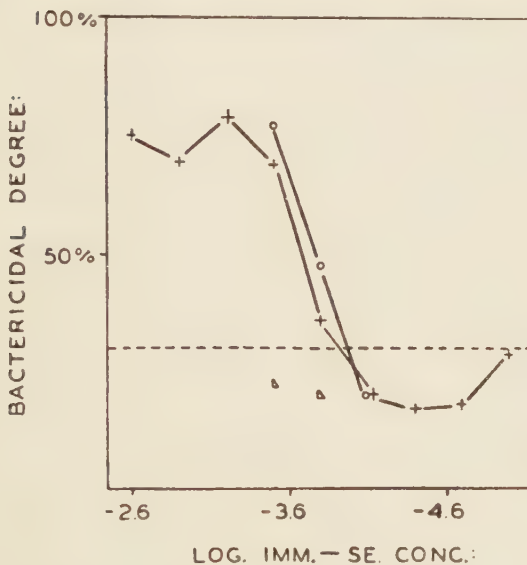
In order further to make sure that bacterial suspensions of normal density are stable in a milieu containing immune body and to examine whether the amount of bacteria plays any great part in the inhibition process a special experiment was made: A bacterial suspension of normal density and one that was 8 times as dense were used for that experiment (Table 25). On interrupting the experiment the difference between the two suspensions was equalised by transferring from the test tubes with a large bacterial dose 0.1 cc. to other test tubes, each containing 7.9 cc. cold saline solution, thus producing a dilution of 1:80 instead of the usual dilution of 1:10 that is used for the test tubes with normal bacterial dose. Both the initial value and the basal effect of the active serum were determined by way of control from both bacterial suspensions and, as referred to on page 53, the passing from normal to 8 times the normal density gave no difference between the percentages of surviving bacteria. The graphic representation of the experiment shows that, both as regards position and steepness, its most characteristic phase—the change from promoting to inhibitory effect—is uninfluenced by the spring from normal to 8 times the normal bacterial density. The most natural conclusion to be drawn from this fact is: That the comparatively dense bacterial suspension is as stable against immune body as the normal one; *and* that at a given concentration of active serum the course of the inhibitory process is independent of the bacterial density within fairly broad limits.

To illustrate the importance of bacteria and immune body being mixed and allowed to stand some time before the experiment is started, the experiment in Table 25 was supplemented with two experimental tubes to which bacteria were not added until all other reagents had been mixed. As might be expected, these additional determinations show that in the transition zone

between promoting and inhibitory effects the usual preliminary treatment of short duration of the bacteria with immune body plays an especially great part. The difference between the results from test tubes with and without preliminary treatment is a little greater than corresponding to the difference between the concentrations of immune body in the period of preliminary treatment and the experimental period (2:1). This seems to indicate that the bacteria actually attain to bind considerable amounts of immune body in the course of the short preliminary

TABLE 25

Bactericidal Experiment with Addition of Immune Serum.



Immune serum: S1 (cf. Table 23).

Curve marked with o: Normal bacterial density.

" " " +: 8 times normal density.

Values " " Δ : Normal density, but without preliminary treatment, (cf. text on p. 88).

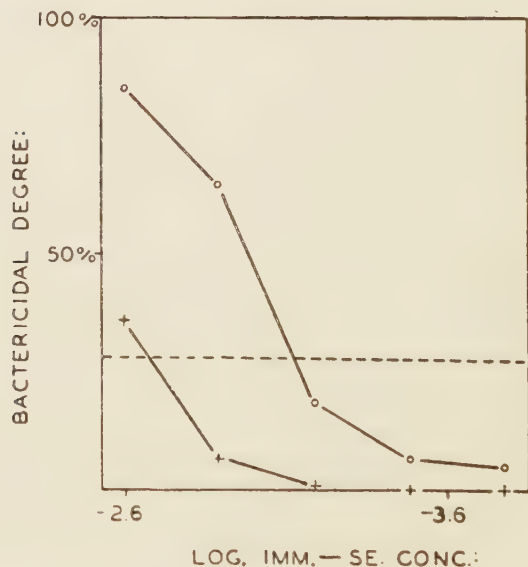
Conc. of active serum 55 %; the dotted line shows the basal effect of the active serum (compare the actual values given in Table 9). As abscissa is used the logarithm of the conc. of immune serum (log. imm.-se. conc.).

Initial value: 184. Experimental culture: Breslau 206.

period, and moreover it seems to indicate that the inhibition becomes most complete when the immune body is bound before the alexin effect sets on.

Just as in the case of normal inactive serum the connection between the effect of the inhibitory substance and the concentration of active serum was examined here. In the experiment, Table 26, the promoting and inhibitory effects of the immune body were examined at two essentially different concentrations of active serum. The largest of the basal concentrations used gave an almost complete bactericide in itself, but if it is considered that the two curves in Table 26 are at highly different levels, it might be taken for proved that the tendency to decline of both

TABLE 26
Bactericidal Experiment with Addition of Immune Serum.



Immune serum S1 (cf. Table 23).

Curve marked with +: Conc. of active serum 55 %.

" " " " " " " " 13.75 %; the dotted line shows the basal effect of this serum conc., with 55 % active serum the survival percentage is less than 1.

Initial value: 344. Experimental culture: Breslau 1A.

curves will reach maximum in the same section of the range examined.* *The inhibitory effect thus seems to begin at a fixed concentration of immune body—independently of the concentration of active serum. The effects of the immune bodies and the inhibitory normal bodies thus also resemble each other in this respect (cf. page 74).*

It has been shown above that in two respects the well-known inhibitory effect of the immune body on the bactericidal process resembles the inhibition that may be produced by means of a large excess of heated serum. It now becomes of special interest to the further comparison between the effects of the thermostable normal substances and the immune bodies to examine whether immune body in a suitable concentration will inhibit the saline-phagocytosis as inactive normal serum did.

Specific immune body has proved to promote phagocytosis within a very broad zone. The total effect measured by the reduction of the number of bacteria in a phagocytosis milieu with active serum + immune serum can be outlined as consisting of three "layers": 1) The spontaneous phagocytosis, limited by the initial value and the phagocytosis value that would be obtained in a control test tube containing blood corpuscles + inactive instead of active serum. 2) The bactericidal effect and the increase of phagocytosis after the inactive serum has been replaced by active serum. 3) The further increase of both effects that is due to the promoting effect of the added immune body.—In case we have a phagocytosis system in which all three "layers" can be distinctly demonstrated, the total phagocytosis effect will generally be very intense and even if the concentration of immune body is highly increased the inhibitory effect that can be produced will still be limited; (cf. the bactericidal experiments, Table 26, showing that even if the concentration of immune body determines the onset of inhibition, the extent of the latter expressed as a percentage will depend on the concentration of active

* A transcription of the experiment by means of probits (see Chapter X), which would render the importance of the percentage decline more conspicuous, could not be used in this case, owing to the very low end-values of one of the curves.

serum). A phagocytosis experiment as the one referred to, in which only a limited inhibition is obtained, even with very high concentrations of immune body, may easily convey the impression that the inhibition only amounts to a discontinuation of the phagocytosis stimulation observed in lower concentrations of immune body and that the inhibition does not interfere with the spontaneous phagocytosis. If the concentration of active serum in the system is reduced, the values of spontaneous phagocytosis and serum phagocytosis will approach each other and the intermediate "layer" will gradually disappear, whilst the phagocytosis can still be stimulated for a long time by means of immune body in suitable concentration. In such a system, where the total phagocytosis effect is not very intense, the inhibitory effect of high concentrations of immune body will be very marked and under suitable conditions a complete extinction of all phagocytosis can be attained.

If we pass on to a milieu that is completely devoid of active serum, i.e. to a test tube containing washed blood corpuscles in

TABLE 27

Phagocytosis Experiment with Addition of Immune Serum.

Immune serum S1 (cf. Table 23).

Experimental culture: Breslau 1, (the experiment carried out together with the phagocytosis experiment in Table 21).

Initial value	Values and degrees of phagocytosis in milieu with,				
	saline solution		active serum 1.25 %		
	without addition	+ S1 10 %	without addition	+ S1 10 %	+ S1 0.25 %
75					
79					
79					
81	80				
82	103	167	94	160	22
101; 83 $\times$ 2 =	113; 99*	175; 171**	118; 106*	163; 162**	24; 23
166	59.6 %	103.0 %	63.8 %	97.6 %	13.8 %

Comparison between the values * 99 and 106 and the values ** 162 and 171 gives $t = 0.74$ and $t = 0.67$ respectively, (calculated from $s^2_{\min.}$).

In spite of the exceptionally high conc. of heterologous serum (10 % rabbit immune serum) nothing abnormal was observed, especially no agglutination of the human erythrocytes was noticed.

saline solution, it appears that here, too, the inhibitory effect of the immune body is conspicuous. *As will appear from the experiment in Table 27, a complete blockade of the saline-phagocytosis and phagocytosis in a milieu with low concentration of active serum was obtained by means of an addition of 10 per cent. immune serum.*

Considering this last observation a comprehensive similarity can be ascertained between the inhibitory effects exercised by the thermostable normal substances in serum and by specific immune bodies. As already referred to in the case of the bactericidal process, it seems to be of special importance to the comparison that specific immune body in high concentration will not only cease to exercise its stimulating effect but that its inhibitory effect will extend to the domains where the normal inactive serum exercises its inhibitory effect, i.e. the basal bactericidal effect and the saline-phagocytosis.

It has now been demonstrated that specific antibody is capable of exercising the double effect referred to in the preceding chapter, as influencing the bacteria, it reduces their phagocytability in saline milieu *and* at the same time their sensitivity to alexin and opsonin. *Owing to the resemblance between the inhibitory phenomena produced by immune body and by normal heated serum it seems natural to identify the inhibitory normal substances with the "normal antibodies".*

As to the mechanism of the inhibitions referred to, there is little to say at present. The original theory of "komplementablengung" advanced by Neisser-Wechsberg (1901) is now considered untenable by most investigators, and among the writer's experiences the fact that there is a continuity between the inhibition in opsonin-containing and opsonin-free milieus goes strongly against the theory. The only conclusion that may be drawn from the experiments described is that inhibition will occur when it is possible for the bacteria to bind large amounts of normal or specific antibody before or at the beginning of the experiment. We still know so little about the interaction between antibody and alexin or opsonin that nothing particular can be said as to *why* an excess of antibody inhibits the antibacterial serum processes. It seems to be more perspicuous that the phagocytability of

a bacterium in opsonin-free milieu can be changed when its surface changes character, which must take place when large amounts of antibody are bound.

In the preceding pages a clear distinction has been made between "normal sera" and such as contain immune body. In reality the distinction is not so easy.

As referred to in connection with the inhibitory effect of the specific antibody on the bactericidal process, there is a great conformity between the content of agglutinin and bactericide-promoting (resp. inhibitory) antibody of a serum. In all the experiments performed by the writer the parallelism was complete so that, if a serum contained agglutinin against one or more antigens in the bacterial strain employed, the same serum proved to be phagocytosis- and bactericide-promoting in experiments with the same strain. In examinations of single colonies with slide-agglutination it has even been possible to demonstrate that a serum for instance containing antibody against one of the H-antigens of the Breslau strains will only stimulate the phagocytosis of the individuals of the bacterial population that contain the antigen in question.

Now, when it is desired to make sure that the "normal sera" employed are free from immune body it will thus be natural first to examine such sera in agglutination experiments with Breslau antigen. It has, however, appeared that the effect of the specific immune body on the antibacterial processes can often be traced in very low concentrations (see Tables 25 and 25); it is thus common for a highly agglutinating serum to be phagocytosis- or bactericide-promoting in concentrations of 1:100,000 or 1:200,000. If these concentrations are compared with the lowest in which the same immune serum is capable of producing agglutination (as a rule about 1:10,000), it will be seen that frequently there must be a concentration interval in which the specific antibody cannot be made out by means of agglutination but is still able to influence a phagocytosis or bactericidal system.

In order to make sure, as best it can be done, that the sera considered as normal do not contain so small amounts of immune body, the "normal" sera employed were examined in a special

manner. In these examinations it was taken for granted that if in a milieu with a high concentration of "normal" serum a great increase of phagocytosis could be brought about by an addition of a small amount of immune serum (e.g. 1: 25,000), the possible content of immune body of the "normal" serum must be so trifling that it can be ignored in practice. The justification of this argument is based on the experience that, if immune body in a concentration of 1: 25,000 produces a great increase of phagocytosis, an addition of 2 or 3 times the amount of immune body will not bring about a further increase of the phagocytosis; the consequence hereof is that the comparatively great amount of "normal" serum in the experimental test tube cannot have contained sufficient immune body to bring up the concentration to anything near 1: 25,000. Thus the content of immune body in sera tested in this manner must be exceedingly slight.

Besides being submitted to Widal's reaction the sera employed in the present work as normal have been tested for content of immune body in the manner stated above.

PART THREE

CHAPTER VI

INTRODUCTION

In the preceding chapters the courses of the bactericidal and phagocytosis processes were examined separately and it was shown that both of these processes are inhibited by an excess of immune body and presumably also by an excess of "normal antibody". On the basis of this experience it is intended in this part to make more direct comparisons between the alexin and opsonin activities in *normal* sera under different external conditions.

Considering the investigations that have hitherto formed the basis of comparisons between alexin, opsonin, and complement the results are often difficult to estimate. The difficulties are first and foremost due to the fact that the three functions—bactericide, phagocytosis and hemolysis—are determined by essentially different methods:

The alexin effect has generally been determined by the serum concentration that after a longer period—2 to 8 hours—gives positively decreased or completely stopped growth. Most frequently (as for instance in Gordon's experiments) the examinations have taken place in a milieu where inoculation from control test tubes gives confluent growth after an experimental period of from 6 to 8 hours. It will hardly play any great part that the action of serum substances on the lag phase and time of division of the bacteria is measured in such experiments besides the bactericide, as increase of lag phase and time of division is doubtless brought about by the very substances that in sufficiently high concentration destroy the germinative power of the bacteria completely (cf. pages 162-163).

The opsonin effect was always previously determined by mi-

microscopical reading of the effect of the fresh serum on phagocytosis. Wulff (1911) strongly emphasized the difficulties in connection with such reading; among other things he ascertained that intraleukocytic bacteriolysis may give rise to erroneous estimation, and as regards the counting itself of phagocytized bacteria Wulff writes that when the films are ready "the most difficult part of the experiment remains". The technical difficulties as regards counting are also traced indirectly in the different "individual" formulae that have been used to give an objective expression of the degree of phagocytosis, see for instance Mørck (1933) and Jersild (1942).

The complement effect is lastly measured as against a sensitized system of sheep- or ox-blood corpuscles, either with determination of the serum dose required for total hemolysis, or of the degree of hemolysis after a given experimental period.

It goes without saying that personal work with all 3 methods throughout a long period is required before comparisons may be drawn between the results obtained by so different means; and it must often be rather coarse, almost qualitative agreements or differences that are observed.

The culture method, as it is described in Part I, however, affords a technique allowing a direct comparison between the intensity of the bactericidal process and that of the phagocytosis process, the two functions being measured in the same milieu, by the same criterion (the disappearance of the living bacterium from the fluid phase) and, therefore, with the same accuracy. On that basis it was probable that the question of the relationship between alexin and opsonin could be taken up again to advantage.

As referred to in the introduction, there is a great resemblance between alexin and opsonin and the only point where the two substances seem to differ is in their relation to Gordon's fourth component. Therefore it is natural first to take up for renewed examination the question of the dependence of the alexin and opsonin processes on the fourth component. Later on a report will be given of investigations in a few domains where direct comparisons between the bactericidal and phagocytosis processes have not previously been made.

The investigations that will be mentioned comprise qualitative and quantitative experiments, which will be dealt with separately. The qualitative experiments were performed as experiments with preliminary treatment, the principle of which is to separate the bactericidal and phagocytosis processes in time and then examine how the two processes are influenced when the serum used for preliminary treatment is dealt with in different ways. The quantitative experiments are made as titrations, some interferences with the same qualitative effect on the alexin and opsonin processes being graded and the whole range of action of the interference being examined simultaneously in the bactericidal and in the opsonin process.

The qualitative investigations will be found in chapters VII-IX; the quantitative investigations in chapters X-XI.

CHAPTER VII

EXPERIMENTS WITH AMMONIA-TREATED SERUM

As referred to in the introduction, Gordon and collaborators have shown through numerous experiments that, when influenced by NH_3 -ions, serum loses its hemolytic and bactericidal power and that serum treated in that manner can be reactivated by heated and in itself inactive serum. The comparatively thermostable component destroyed by ammonia is generally termed the fourth component of the complement. Gordon's observations were later on confirmed by Deissler, Ecker *et alii*.

Gordon & Wormald (1928) found that the hemolytic and bactericidal processes behave alike as regards the fourth component and this observation is one of the many ponderous arguments in favour of the perception that the hemolytic and bactericidal agents—i.e. complement and alexin—are identical. As to opsonin, the matter is less clear.

Gordon, Whitehead & Wormald, in a publication of 1926, conclude that opsonin differs from alexin in that it does not require the concurrence of the fourth component.—In a subsequent publication by the same authors (1929) they first emphasized the numerous examinations that have demonstrated a close relationship between the alexin and opsonin processes and then detailed two experiments from which they concluded that NH_3 -treatment of serum does not influence the opsonin process. This conclusion is, however, open to objection; and as the publications referred to above have been quoted by several authors—Muir (1931), Osborn (1937) and Jersild (1942)—as a proof of the assertion that complement and alexin differ from opsonin, it will be necessary to give detailed reasons why the writer considers this allegation insufficiently grounded.

Gordon and collaborators used a system of sensitized ox blood corpuscles for the experiments and stated that 1.0 and 0.5 cc. guinea-pig serum diluted 1:10 gave total hemolysis. Unfortunately no hemolysis titration is stated, but if it is taken for granted that the 0.5 cc. is near the requisite hemolytic dose, 0.1 cc. of the dilution stated may be reckoned not to give any perceptible hemolysis (e.g. it applies to hemolysis of sheep's blood corpuscles that if the requisite hemolytic dose is reduced to about $\frac{1}{4}$ little or no hemolysis is obtained).—In the said work Gordon used a hypertonic NH_3 -solution for the ammonia treatment, so that after the treatment the serum is only slightly diluted. The reactivation was brought about by mixing equal parts of heated and NH_3 -treated serum and it is stated that in this mixture the hemolytic power was completely restored.—The opsonin activity was determined by means of phagocytosis experiments with the staphylococcus aureus: 0.1 cc. bacterial suspension was mixed with 0.5 cc. serum (undiluted guinea-pig serum) and incubated for 1 hour at 37° ; after addition of washed leukocytes renewed incubation for 1 hour at 37° , whereupon films were made. The result is stated as good or no phagocytosis and it is stated that further differentiation by means of counting of phagocytized bacteria was unnecessary.—Gordon's first experiments showed that both fresh and NH_3 -treated serum produced good phagocytosis; under the given experimental conditions NH_3 -treatment of serum thus did not perceptibly influence the opsonin activity against the staphylococcus aureus.

On looking at the quantitative conditions it is, however, seen that an NH_3 -treatment that is sufficient to discontinue the hemolysis need only have reduced the serum activity to about 10 per cent., i.e. the phagocytosis experiment *may* have taken place in a milieu corresponding to one with serum diluted in the ratio 1:10. Considering the long duration of the experiment and the great bacterial density, undiluted serum and serum diluted in the ratio 1:10 may readily have produced uniform and marked phagocytosis.

In the second experiment reported by Gordon he used guinea-pig serum absorbed with large quantities of the staphylococcus aureus. It is shown that this serum had lost all hemolytic and

opsonic activity, while it was still capable of reactivating NH_3 -treated serum. At the same time it is shown that the absorption bacteria had been influenced so as to be more readily phagocytized than normally. It was concluded from the experiment that the stimulation of the phagocytosis is due to opsonin being bound to the bacteria in the course of absorption, the serum thus having lost its opsonin activity, and that the fourth component remaining in the absorbed serum therefore is not required for the opsonin effect.

Two objections can be made against this conclusion:

First, there is a great difference between the concentration of the thermolabile components required for a given serum activity and that of the fourth component. Absorption of for instance 99 per cent. of the thermolabile substance will probably result in distinctly decreased phagocytosis measured in Gordon's system, while the dose of the fourth component required for partial reactivation is so small that in the course of an experiment of long duration one may run the risk of allowing even a 99 per cent. disappearance of the fourth component to pass unnoticed (it is especially hazardous in this connection to use a large and constant reactivation dose as Gordon does, judged by the brief report of the experiment). As to the necessary reactivation dose of the fourth component, see the quantitative experiments page 155.

The second objection is of fundamental nature: It cannot be considered proved that the fourth component has been inactive in the course of the opsonin influence on the absorption bacteria, even if it remains in the serum after the absorption. In complex processes several instances are known of co-factors taking part in a reaction without being spent, and according to Deissler (1932) it is said to apply just to the fourth component that, although necessary to the hemolytic process, it is not consumed, or at any rate only slightly so.

In a more recent publication Gordon & Thompson (1935) reverted to the question of the importance of the fourth component to the opsonin process. In that publication the authors examined the decline of hemolytic and opsonic activity, respectively, occurring when serum is treated in different ways: When

allowed to stand at room temperature; on addition of Congo red; on treatment with NH_3 ; on addition of hypertonic K- or Na-salts or change of the pH. It is stated that these five interferences all of them reduce both the hemolytic and the opsonic activity and that the hemolytic activity is always discontinued first, while a more intense treatment of the serum is required before the opsonic activity is influenced or discontinued.—Gordon & Thompson's conclusion: That presumably there is a qualitative difference between complement and opsonin, does not seem to be quite obvious. It cannot be ignored that the difference between complement and opsonin observed by Gordon & Thompson may be an outcome of a difference of sensitivity between the methods of measuring used. Supposing for instance that the phagocytosis system employed is sensitive to weaker serum concentrations than the hemolytic system is (cf. the very long periods of preliminary treatment and phagocytosis in the experiments referred to above), Gordon & Thompson's observations rather seem to indicate close qualitative correspondence between complement and opsonin.

WRITER'S EXPERIMENTS

After this review of Gordon's experiments it seems just to maintain that they give no decisive reply to the question of the dependence of the opsonin process on the fourth component. In a fresh attempt at comparing the two substances a closer examination of the alexin and opsonin activities in NH_3 -treated serum will, therefore, be much needed.

All the Gordon experiments referred to were made with guinea-pig serum. To venture a comparison with his own experiments the writer repeated, with human serum, Gordon's classical inactivation and reactivation experiments.—The experiment, Table 28, shows that Gordon & Wormall's observations concerning the alexin process and its dependence on the fourth component also applies to human serum. As will be seen from the experiment there remained a slight, not quite safe alexin effect after the NH_3 -treatment. The destruction of the fourth component

TABLE 28

Bactericidal Experiment with Ammonia-treated, Human Serum.

The solutions of NH_3 and HCl used are isotonic with the normal saline solution. Serum treated in the following ways is employed:

- a) fresh serum incubated for 60' at 37° .
- b) (1 part of serum + 0.25 parts of NH_3 + 0.25 parts of HCl) incubated for 60' at 37° , (control serum).
- c) (1 part of serum + 0.25 parts of NH_3) incubated for 60' at 37° , + 0.25 parts of HCl , (i.e. " NH_3 -treated serum").
- d) Serum incubated for 30' at $55\text{--}56^\circ$.

The total experimental volume in this case 1.4 cc. including the bacterial suspension. Experimental culture: Breslau O-form.

Experimental milieu	Bactericidal values	Bactericidal degrees
Saline solution (initial value)	160 160 162 192; 169	
Serum d 0.6 cc. + saline 0.6 cc.	156 160 168 184; 167	168* 100.0 %
Serum a 0.6 cc. + saline 0.6 cc.	40 42 46 48; 44	26.2 %
Serum b 0.9 cc. + saline 0.3 cc.	21 29 41; 30	17.8 %
Serum c 0.9 cc. + saline 0.3 cc.	147 152 154 157; 152*	90.8 %
Serum c 0.9 cc. + „ d 0.3 cc.	39 50 51 62; 51	30.1 %

Comparison between the values * 168 and 152 gives $t = 1.78$ and $t = 1.94$, calculated from s^2_{max} and s^2_{min} respectively.

A hemolysis experiment performed simultaneously showed inactivation and reactivation exactly corresponding to what was found in the bactericidal experiment.

was, however, fairly complete both in this and in the subsequent experiments, and it was always possible to carry through distinct reactivation by means of heated serum.

The *experiments with preliminary treatment* used in this chapter and the following ones for the qualitative examinations are performed according to the following principle:

For the preliminary treatment a series of mixtures of the same bacterial density is used; the latter is preferably made about 1000 times the density of the bacterial suspensions from which bacteria are generally drawn for the experimental test tubes. The following mixtures may for instance be used for a typical experiment:

- 1) Bouillon culture + saline sol. (control experiment)
- 2) " " + " " + inactive serum
- 3) " " + " " + fresh serum
- 4) " " + fresh serum + a solution of the substance the effect of which on the alexin and opsonin processes it is desired to examine.

The three last mixtures are incubated at 37° for a suitable time (dependent on the alexin sensitivity of the strain employed). At the end of the incubation period mixture No. 1 is prepared for the control experiment and then 0.1 cc. is transferred from each of the mixtures Nos. 1-4 to bottles, each containing 100 cc. saline solution. This dilution in the ratio 1:1000 partly causes the serum processes to be interrupted, partly reduces the serum concentration so much that the presence of serum plays no part in the further course of the process* and, lastly, the bacterial density is reduced to a suitable level.—As will be seen, the dilution with which the preliminary treatment is concluded takes the place of the time-wasting washing and centrifugation used by previous investigators for the bacteria submitted to preliminary treatment. By means of the dilution procedure it is also rendered

* That the diluted serum is actually indifferent appears from control determinations, in which the dilution for the control experiment was made with weak serum-saline of such a concentration that the same concentration of fresh serum was obtained in the tubes for the control experiment as in the experimental tubes, to which a small amount of fresh serum is transferred from the milieu of preliminary treatment (cf. Table 32).

possible to examine the properties of the living bacteria *immediately after the cessation of the preliminary treatment*, which was not possible formerly.

The bacteria treated in different ways are transferred to the experimental test tubes proper from the weak bacterial suspensions in the bottles: From each bottle 0.2 cc. is added to an experimental test tube containing 0.8 cc. saline solution, and the same volume is added to a phagocytosis tube containing 1.25 cc. suspension of washed blood corpuscles in saline solution. These test tubes are used for a general determination of the degree of saline-phagocytosis of the bacteria submitted to preliminary treatment; the ultimate result of the experiment is estimated in the following manner:

a) From mixture No. 1 (control experiment), with no preliminary treatment of the bacteria which have just passed through the same series of dilution as those submitted to preliminary treatment, the initial value of the experiment and the corresponding value of the saline-phagocytosis are determined.

b) From mixture No. 2 (bacteria + inactive serum) values are determined that will very nearly coincide with the values of the control experiment. The great dilution finishing the preliminary treatment will render the inhibitory effect on phagocytosis of the inactive serum practically imperceptible (cf. page 79).

c) From mixture No. 3 (bacteria + fresh serum) the number of surviving bacteria is determined, and on comparison with the initial value of the control experiment an exponent of the bactericidal degree in the milieu of preliminary treatment is arrived at. In addition a saline-phagocytosis value is determined which, on comparison with the number of surviving bacteria (the initial value of the c-experiment), gives an exponent of the phagocytability of the latter.

d) From mixture No. 4 (bacteria + fresh serum + agent) again two values are determined: From the first of them the bactericidal degree in mixture No. 4 is computed and on comparison with the bactericidal degree in mixture No. 3 it is decided whether the agent that has been added influences the alexin process. From the second value the degree of saline-

phagocytosis for the surviving bacteria is computed in the manner stated above, and on comparison between the degrees of phagocytosis in the c- and d-experiments it is decided whether the agent has influenced the opsonin process.

The experiment, Table 29, represents a qualitative experiment made according to the above principle. Instead of addition of an extraneous agent the fresh serum was here treated with NH_3 , and what is examined is, therefore, the effect of the NH_3 -treatment on the alexin and opsonin processes.

It appears from the experiment that preliminary treatment in fresh serum both causes about 33 per cent. of the bacteria to be killed and the surviving bacteria to be more readily phagocytized than those that have not been submitted to preliminary treatment (32.8 per cent. free bacteria as compared to 75.0 per

TABLE 29

Experiment with Bacteria preliminarily treated with Fresh or Heated Serum or with Serum treated with NH_3 or Ether.

Heated serum and NH_3 -serum prepared as described in Table 28; ether-treated serum prepared as follows: Equal volumes of fresh serum and ether are shaken together for about 1 min. after which the ether is removed with pipette and through evaporation. The serum used smelt slightly of ether and attempts at reactivation with heated serum only succeeded partially, (cf. text on p. 112).

Experimental culture: Breslau 1.

Preliminary treatment	No treatment	Treatment for 30' at 37° in			
		heated serum 50 %	NH_3 -serum 75 %	ether-serum 50 %	fresh serum 50 %
Number of bacteria surviving treatment (initial values):	352		352	347	241
	356		344	349	238
	378; 362		317; 338	353; 350	243; 241
	100.0 %		93.4 %	96.6 %	66.6 %
Values and degrees of saline phagocytosis:	253	272	262	259	78
	269	276	265	272	79
	290; 271	300; 283	269; 265	313; 281	80; 79
	75.0 %	78.2 %	78.4 %	80.2 %	32.8 %
	of 362	of 362	of 338	of 350	of 241

cent.). The experiment moreover shows that *preliminary treatment in NH_3 -serum neither leads to bactericide nor to stimulation of phagocytosis*. The experiment thus seems to indicate that both the alexin and the opsonin process require the concurrence of the fourth component.

Gordon's assertion that opsonin acts independently of the fourth component is thus hardly correct, which in fact has been shown, though not commented on, in the experiment, Table 20, in which it was seen that NH_3 -treated serum inhibits phagocytosis just like heat-inactivated serum. According to Gordon's theory NH_3 -treated serum should, on the contrary, have promoted the phagocytosis highly.

A question of fundamental importance, not only to the experiments with preliminary treatment, will be mentioned here, as it was examined by means of the technique described above. It is the question whether the bactericide and the phagocytosis stimulation will cease completely on interruption of the experiment, or whether there is an "after-effect" similar to the oft-mentioned "after-hemolysis".—A possible protracted bactericide would, of course, render it very difficult to carry through large experiments, as a change of the number of bacteria within the inoculation period would have to be reckoned with. As referred to on page 40, cultivations from the same test tube at different times after the interruption of the experiment showed that the number of bacteria remains constant, at any rate within the first hour. It will be shown in the following experiment that not only the alexin influence but also the opsonin influence to which the bacteria are submitted in the course of the preliminary treatment will cease on dilution of the serum.

In the experiment, Table 30, samples for determination of percentage of survival and phagocytability were taken 3 times at intervals of half an hour after the preliminary treatment had been interrupted. The experiment shows that conditions remain constant within 1 hour after interruption of the preliminary treatment. This again means that *both* the alexin and the opsonin processes are discontinued completely when the fresh serum is diluted in the ratio 1:1000.

(Theoretically, the following objection could be raised against the conclusions drawn from experiments with preliminary treatment as those described above: It cannot at first glance be seen from the experiments whether the increased phagocytosis after preliminary treatment in fresh serum is due to the fact that the bacteria as a whole have become more phagocytal, or whether the surviving bacteria are just a selection of individuals that are especially readily phagocytal in saline milieu. Now, the latter supposition is in itself improbable, and as to the experiment,

TABLE 30

Experiment with Preliminary Treatment, set up for Demonstration of a possible "After"-effect.

From the suspensions of treated and untreated bacteria (i.e. from the dilution bottles) 3 withdrawals were made and the numbers of surviving bacteria and the degrees of saline-phagocytosis were determined at intervals of 30 min.

Experimental culture: Breslau 1.

Time of withdrawal	No preliminary treatment		Treatment for 30' at 37° in fresh serum 67 %	
	Numbers of bacteria	Values and degrees of saline-phagocytosis	Numbers of surviving bacteria	Values and degrees of saline-phagocytosis
0 min.	514	275	219	28
	525	280	231	30
	550; 530	269; 275	233; 228	49; 36
		51.1 %	42.4 %	15.8 % of 228
30 min.	526	294	228	25
	536	301	230	40
	566; 543	304; 300	246; 235	41; 35
		55.7 %	43.7 %	15.4 % of 228
60 min.	523	315	207	47
	538	320	223	48
	560; 540	354; 330	231; 220	53; 49
		61.3 %	40.9 %	21.5 % of 228
Averages:	538 = 100 %	301 = 56.0 %	228 = 42.5 %	40 = 17.5 %

Comparison of the three mean values in columns 1-4 gives $P = 90.95 \%$, $P = 7 \%$, $P = 90.95 \%$ and $P = \text{about } 3 \%$ respectively.

Table 29, it will be seen that, even if all the bacteria killed in the course of the preliminary treatment (121) were supposed to be non-phagocytizable in saline milieu, this supposition alone cannot explain the results. Among the bacteria treated in fresh serum there were 79 unphagocytized, which together with the 121 would give 200 unphagocytized bacteria as compared to the about 270 unphagocytized bacteria found in the control experiment).

On going through the literature it was stated that shaking with ether or chloroform inactivates serum and that, presumably, it is the fourth component that is destroyed. As shown by Tokano (1936), the ether treatment has, however, the drawback that reactivation of the treated serum is possible only if the intensity of the treatment has not exceeded a certain limit, which cannot be fixed in advance as applying to all sera. If the proper degree of ether treatment of the serum is successfully arrived at, the inactivated serum can be reactivated by heated serum, just as is the case after NH_3 -treatment. Owing to the fickleness of the ether method Gordon's classical method has almost exclusively been used in the investigations into the function of the fourth component in the present publication.

A few experiments were, however, made with ether-treated serum; e.g. in connection with the experiment, Table 29. It appears from the values for ether-treated serum that it behaves just like NH_3 -treated serum, as it neither shows alexin nor opsonin activity.

CHAPTER VIII

EXPERIMENTS WITH ANTICOMPLEMENTARY SERUM

The concept of anticomplementary serum (or self-inhibitory serum) is confined here to comprise certain rare human sera that have a distinct capacity of binding complement without the concurrence of extraneous antigen. Sera of this special nature and of the characteristic strength are found in some patients with high serum globulin values, especially in patients suffering from myelomatosis (Jersild, 1936 and 1939).

In previous publications on the anticomplementary effect of serum, effects of rather frequent occurrence but faint, too, are described, which shows that the investigators in question (Liefmann & Stutzer, 1910, Mackenzie & Marshall, 1932, Auguste, 1934, *et al.*) have not worked with myeloma sera which would have segregated solely owing to their strength. A myeloma serum of medium strength will discontinue the hemolytic effect when the ratio between anticomplementary serum and complement (guinea-pig serum) is about 1:125, while the faintly anticomplementary sera referred to will not be effective until the ratio to complement is about 1:5.

As to the NH_3 -treatment dealt with in the preceding chapter, the manner in which it influenced complement and alexin was known beforehand; or rather, after exclusion of certain effects, a new component was defined as the factor that is destroyed by ammonia under certain fixed conditions. As nothing is known with certainty in advance about the mode of action of the anticomplementary serum the mechanism of the inhibition will first be briefly discussed.

Strangely enough the anticomplementary effect of myeloma sera will only appear after heat treatment, and genuine serum is

generally rich in complement (see Table 31 at the bottom). This seems to indicate that in the course of the heat treatment transformations or re-arrangements take place in the protein molecules to which the anticomplementary effect is attached and that it is denatured protein that combines with complement (Jersild, 1936). It might, of course, also be imagined that the pathologic proteins of a myeloma serum possessed the anticomplementary property before the inactivation and that in the genuine serum they were supersaturated with complement, which would thus occur both in free and in conjugate form. Jersild's experiments with inactivation at different temperatures, however, speak greatly in favour of the former view (Jersild & Pedersen, 1938).

From theoretical considerations Jersild (1942) tried to use anticomplementary serum as an addition to defibrinated blood for phagocytosis experiments. In this manner he succeeded in impairing the normal phagocytosis so much as to make the immune phagocytosis manifest itself clearly.—A few phagocytosis experiments with addition of weak anticomplementary sera were previously made by Dean (1907).

WRITER'S EXPERIMENTS

The writer has only made a few experiments of his own to elucidate the mode of action of anticomplementary serum. It will be seen from the experiment, Table 31, that if an anticomplementary serum is supersaturated with complement (guinea-pig serum) and, after a suitable fixation period, is inactivated by means of heat treatment, the anticomplementary serum in the mixture will again be capable of combining with complement. Still, only a fixation of between 20 and 33 per cent. of what was first obtained under similar conditions was achieved the second time. It has appeared that concentrations are of considerable importance to the fixation capacity; the latter will thus be greatest when undiluted sera are mixed, while in case the complement is diluted as in an ordinary hemolysis experiment, a far smaller amount of complement is fixed per cc. anticomplemen-

tary serum. On dilution of a mixture of anticomplementary and fresh serum it will, therefore, be possible that the effect of the dilution is counteracted by the splitting off of fixed complement; this may be the reason why the turning point of the titrations in Table 31 are so indistinct as compared with common hemolysis experiments.

It will also appear from Table 31 that the extent of the re-established capacity to combine is independent of the duration of the heat treatment. This corresponds to conditions in the

TABLE 31

Hemolysis Experiment with Mixtures of Fresh Guinea-pig Serum and Anticomplementary Serum.

The following mixtures were employed:

- I: Anticomplementary serum* 0.03 cc. + fresh guinea-pig serum 12.0 cc.
 II: " " 0.01 cc. + heated " " 4.0 cc.
 III: Normal heated serum 0.01 cc. + fresh " " 4.0 cc.

Preliminary determination of the hemolytic power of the mixtures:

Mixture I in cc.	Degrees of hemolysis	Mixture III in cc.	Degrees of hemolysis
0.1	60 %	0.0125	100 %
0.05	50 %	0.006	100 %
0.025	20 %	0.003	90 %
0.0125	0 %	0.0015	50 %
		0.0008	10 %

The three mixtures were incubated for 30' at 37° and then inactivated by heating to 56°, (mixture I was split in three equal portions which were heated for 15, 30 and 60' respectively, mixtures II and III were heated for 30').

After the inactivation 6 times 0.5 cc. were taken from each mixture and to these portions varying amounts of fresh guinea-pig serum were added. The tubes with the new mixtures were incubated for 30' at 37° and their hemolytic power was subsequently determined. For this determination 0.1, 0.05 and 0.025 cc. were taken from each tube, 0.3 cc. of saline and 0.2 cc. of a suspension of sheep erythrocytes (a 2.5 % suspension sensitized with rabbit amboceptor) added and the tubes placed in an incubator at 37° for 60'. The experiment was read next morning, the degrees of hemolysis being stated in per cent. of the total hemolysis.

The three portions of mixture I inactivated for different periods gave identical results; consequently but one of these experiments is reproduced here, (on top of next page).

TABLE 31 (cont.)

Amount of fresh guinea- pig serum added to 0.5 c. of mixtures I-III	Fresh guinea-pig serum +								
	mixture I (inactivated)			mixture II (inactivated)			mixture III (inactivated)		
	0.1	0.05	0.025	0.1	0.05	0.025	0.1	0.05	0.025
0.02 cc.	0	0	0	—	—	—	90	70	20
0.03 cc.	0	0	0	—	—	—	100	90	60
0.045 cc.	10	0	0	—	—	—	100	100	90
0.07 cc.	30	30	10	0	0	0	100	100	100
0.10 cc.	60	50	20	0	0	0	100	100	100
0.15 cc.	90	80	60	0	0	0	100	100	100
0.23 cc.	—	—	—	20	20	10	—	—	—
0.34 cc.	—	—	—	60	60	30	—	—	—
0.50 cc.	—	—	—	90	90	70	—	—	—

* Before inactivation the anticomplementary serum employed held between 100 and 200 hemolytic doses of complement pr. cc.

primary inactivation, the duration of which is also without any great importance to the anticomplementary strength obtained by the serum.—The results of the experiments are most easily explained if it is supposed that *one or both of the thermolabile components of the complement will combine with substances in the anticomplementary serum.*

On the basis of Jersild's experiments, which very clearly show the inhibitory effect of anticomplementary serum on the phagocytosis process, it was natural to try to use the strong anticomplementary sera in the present investigations. In all the experiments it appeared that an inhibition of the bactericidal process corresponded to the inhibition of phagocytosis demonstrated by Jersild.

This qualitative correspondence is seen distinctly from an experiment with preliminary treatment made along the same lines as the experiments referred to in the preceding chapter.

On comparison between bacteria treated in fresh serum and such as have been treated in fresh + anticomplementary serum it appears from Table 32 that *both the alexin and the opsonin processes are highly influenced by anticomplementary serum.* The latter is thus capable of discontinuing the alexin effect, which

manifested itself by a percentage of survival of 53, and the opsonin effect, which reduced the percentage of free bacteria from 66 among the untreated to 15 among the surviving bacteria.

TABLE 32

Experiment with Bacteria preliminarily treated in Fresh Serum and in Fresh + Anticomplementary Serum.

The values for untreated bacteria were determined from suspensions of bacteria in diluted serum (1:1500), thus bringing about the same conc. of fresh serum in all test tubes (cf. text on p. 107). Moreover controls were made showing that the last portion of rinsing water from the washing of the blood corpuscles had no bactericidal effect.

The experiment was made together with that in Table 37; the first and third column in the two Tables therefore showing identical figures.

Experimental culture: Breslau 1.

Preliminary treatment	No treatment	Treatment for 30' at 37° in	
		fresh serum 67% + anticomplementary serum 16.7%	fresh serum 67% + saline
Number of bacteria surviving treatment (initial values):	219	200	116
	230	210	120
	234; 228	213; 208	124; 120
	100.0 %	91.2 %	52.6 %
Values and degrees of saline-phagocytosis:		135	14
	147	143	15
	155; 151	151; 143	25; 18
	66.2 % of 228	68.7 % of 208	15.0 % of 120

CHAPTER IX

CITRATE, OXALATE, AND TARTRATE EXPERIMENTS

It appears from the literature that the inhibitory effect of different salts on phagocytosis has been known for a long time. Hektoen & Ruediger (1905) and Wright & Reid (1906) showed that hypertonic salt solutions reduce the phagocytosis; the former believed that probably the inhibition was due to decreased serum activity, whereas later investigators (Sauerbeck quot. from Jersild 1942) believed it was due to injury to the leukocytes. Huddleson & collaborators, who used citrate blood for their diagnostic phagocytosis determinations, showed that addition of 0.5 per cent. of citrate inhibited the normal phagocytosis suitably so that the stimulating effect of the immune substances was very clearly recognized. In most recent times Jersild (1942) has described the inhibitory effect of citrate—both on normal and immune phagocytosis.

Jersild demonstrated the citrate inhibition (as well as the inhibition caused by anticomplementary serum) by means of microscopy experiments with the *Brucella* ab. Bang; his observations, therefore, seem to indicate that the inhibitory effect on phagocytosis of citrate (and the inhibition caused by anticomplementary serum) is of a more universal character than might be gathered from the present publication, in which the effects are only examined against the *salmonella* Breslau.

The effect of citrate on the hemolysis process is also well-known. It is a general experience that citrated blood may not be suitable for complement fixation experiments, because the requisite hemolytic complement dose will increase when the citrate concentration of the system exceeds a certain limit.

There is also a number of communications about the effects

of different salts on the bactericidal process. Special investigations into the effect of citrate and oxalate have only been found by the writer in a publication by Pettersson (1902), whose results will be dealt with on page 137.

In all it is thus known that citrate influences the phagocytosis and hemolysis processes and possibly also the bactericidal process, but it will not be ventured to say anything certain about the mechanism of the influence in the individual cases. Just as in the case of anticomplementary serum it will, therefore, first be tried to elucidate the mode of action of citrate through special experiments.

WRITER'S EXPERIMENTS

An addition of citrate must be imagined in advance to be able to influence the properties of the reaction milieu in at least 3 different ways: a) By a displacement of the pH; b) by a change of the osmotic pressure; and c) owing to a specific influence of the citrate on the active serum substances.

re a) The Na-citrate used is the normal salt with $5\frac{1}{2}$ mol. water of crystallization and in the concentrations generally used it has a pH of about 7.40, i.e. very near the normal pH of the blood. Compared with the experience of previous researchers showing that the complement process has a relatively broad pH optimum with pH between about 6 and about 8 (Michaelis & Skwirsky, 1909, Osborn, 1934) the possible slight effect of the citrate on the pH of the experimental milieu must be supposed to be without any importance.

re b) The phagocytosis experiment, Table 33A, shows how the serum activity is influenced by addition of citrate, oxalate, and tartrate and by a hypertonic NaCl solution. It is clearly seen that the characteristic great inhibitions—changes from about 2 to between 36 and 67 per cent. free bacteria—are produced only by citrate and oxalate.—It is known from investigations into the hemolysis process that additions of hypertonic neutral salts inhibit the complement effect moderately and, as referred to above, hypertonic solutions may possibly also decrease the phagocytic

power of the leukocytes. Some combination of these two effects must presumably be the cause of the modest inhibition seen on addition of the hypertonic solutions of tartrate or NaCl. Compared with the citrate and oxalate effects the latter inhibitions—changes from 2 to about 5 per cent. free bacteria—are quite insignificant.

It is not known with certainty whether the slight increase of osmotic pressure produced by addition of small amounts of hypertonic citrate solution really reduces the phagocytic power of the leukocytes. The question has not been examined more closely, as control experiments have shown that the increase of osmotic pressure accompanying the citrate additions are without any importance to the inhibition of phagocytosis attained.

TABLE 33A

Comparison of the Inhibitory Effect on the Serum Activity produced by Citrate, Oxalate, Tartrate or Hypertonic Solution of NaCl.

3 % solutions of the normal Na-salts of the acids mentioned were used together with a 3 % solution of NaCl; the pH of the solutions preliminarily adjusted to about 7.4.

Experimental culture: Breslau 1. Rabbit blood.

Initial value: 356.

Values and degrees of total serum activity in fresh serum 55% ₁₀ + the following additional reagents		
Reagent	Experimental values	Experimental degrees
Saline	6; 6	1.7 %
Na-citrate	128	
10 %	134; 131	36.5 %
Na-oxalate	171	
10 %	174; 173	48.7 %
Na-oxalate	230	
20 %	248; 239	67.1 %
Na-tartrate	8	
10 %	11; 10	2.8 %
Na-tartrate	14	
20 %	18; 16	4.5 %
NaCl (3 % sol.)	16	
20 %	18; 17	4.8 %

Considering the volume of the citrate additions a 10 per cent. solution of the sodium citrate referred to is generally used. This solution is rather highly hypertonic, and for the control experiments referred to above it proved desirable also to have a citrate solution that was isotonic with serum. As it is no easy matter to decide on the citrate concentration that will be exactly isotonic with the fluid phase of the experiments, considering dissociation conditions in serum- and NaCl-milieus, the preparation of the Pharmacopea danica, "sol. Na-citratiss pro transfusione", was chosen as a useful approach to the ideally isotonic solution. A simple computation shows that this approach is fairly good; if the sodium citrate of the pharmacopea (containing only 1 mol. water of crystallization) is imagined to be completely dissociated, a 2.14 per cent. solution will be isotonic with 0.9 per cent. saline solution; the solution of the pharmacopea contains 2.5 per cent. citrate, so it can hardly be hypertonic but will sooner be slightly hypotonic owing to incomplete dissocia-

TABLE 33B

Comparison of the Effects on Coagulation produced by Citrate, Tartrate or Hypertonic Solution of NaCl.

The salt solutions described above were used in this experiment too.

Experimental technique: Immediately after it was drawn by heart puncture a portion of rabbit blood was quickly distributed with 3 cc. in each of the test tubes; in the tubes were prepared mixtures of saline and the different salt solutions, intended on addition of the blood to give total volumes of 5 cc. and the desired salt concentrations. The tubes were rapidly corked, their contents gently mixed and the time for complete coagulation determined, (at room temperature and with readings every min.).

Rabbit blood 60% with addition of	Time of coagulation at 16°
Saline solution 40 %	12 min.
NaCl (3 % sol.) 40 %	120-150 min.
Na-citrate 10 %	no coagulation within 24 hours
Na-tartrate 10 %	21 min.
Na- " 20 %	30 "
Na- " 40 %	70-80 min.

tion. If the difference in content of water of crystallization is considered a 3.2 per cent. solution of the more aqueous preparation employed here will correspond to the 2.5 per cent. solution of the pharmacopea.

If the 3.2 per cent. solution is considered isotonic with serum + saline solution, an addition at random of this solution will only influence the osmotic pressure in an experimental test tube slightly. As the total volume in the experimental test tubes is kept constant by means of saline solution *there will thus be a certain difference between the osmotic pressures in two test tubes with the same citrate concentration but with additions from the 3.2 per cent. and 10 per cent. solution respectively.* This difference is due to the excess volume of saline which the test tube with an addition of 10 per cent. citrate must contain over that with an addition of 3.2 per cent. when the total volume has to remain constant.—If it is taken for granted that an addition of 3.2 per cent. citrate will not influence the osmotic pressure perceptibly, the difference between the pressures in the above test tubes containing citrate will be equal to the difference between the pressure in a citrate-free test tube and that in the tube containing an addition from the 10 per cent. solution.

The 3.2 per cent. and 10 per cent. citrate solutions were used for the experiment, Table 34, and from each solution one experimental test tube was prepared with 0.45 per cent. citrate and one with 0.675 per cent. citrate. Comparisons between the reactions in these four test tubes plus a citrate-free control test tube show that solely the citrate concentrations in the test tubes determine the degree of inhibition. The difference between the osmotic pressures in the citrate-free test tube and the test tubes with an addition of "isotonic" citrate solution on the one hand and the pressures in the test tubes with an addition of the hyper-tonic 10 per cent. citrate solution on the other hand did not influence the results of the experiments.

According to the above, the changes of osmotic pressure met with in the experiments must be considered too small to influence the serum activity or the phagocytic power of the leukocytes. As it is moreover known from control experiments that neither citrate nor oxalate in the concentrations employed inhibit the

spontaneous phagocytosis, neither of the two salts can possess any specific poisonous effect on the leukocytes. To arrive at an explanation of the citrate inhibition the possibility will now be examined that is stated under c): That citrate (and oxalate) exercise a specific inhibitory effect on the serum activity.

On considering the mechanism of the citrate and oxalate inhibition it suggests itself to think of the inhibitory effect of those substances on another thermolabile process in the blood—namely coagulation. It is now universally accepted by coagulation investigators that the inhibitory effect on coagulation of citrate, oxalate, and sulphate, too, is due to their combining with the free serum-Ca.—In this connection it can be mentioned that in the opinion of several investigators there is probably also a certain dependence between the complement and alexin processes and the Ca-content of the serum: Gordon, Whitehead & Wormal (1926) thus found that the non-diffusible Ca was of importance to the power of different serum fractions to reactivate NH_3 -treated serum. Wadsworth, Maltaner & Maltaner (1936), on the other hand, stated that it was ionized Ca that took part in the alexin process. Pillemer & Ecker (1941), who worked espec-

TABLE 34

Experiment designed to show a possible Inhibition of Phagocytosis through Rise of Osmotic Pressure on Addition of Citrate.

For the addition of citrate, solutions of normal Na-citrate of 3.2 and 10 % respectively were used, (cf. text on p. 121).

The conc. of fresh serum 35 % in all phagocytosis-tubes.

Experimental culture: Breslau 206.

Initial value	Values and degrees of phagocytosis in fresh serum +				
	saline	0.45% Na-citrate from solutions of		0.675% Na-citrate from solutions of	
		3.2 %	10 %	3.2 %	10 %
37					
46		28	27	54	56
52	0	37	36	54	62
57; 48 × 2	0; 0	46; 37*	37; 33*	67; 58**	71; 63**
96	0.0 %	38.6 %	34.7 %	60.7 %	65.6 %

Comparison of the values * 37 and 33 and the values ** 58 and 63 gives $t = 0.76$ and $t = 0.74$ respectively as calculated from $s^2_{\min}$.

ially with problems concerning the fourth component, believe that Ca is a constituent of the pseudoglobulin complex in which the fourth component is contained, still without being necessary to its function.

In order more closely to examine the nature of the citrate and oxalate inhibition the writer's investigations were supplemented with experiments with addition of tartrate. As referred to on page 120, the experiment, Table 33A, showed that tartrate does not produce inhibitions of the same magnitude as citrate and oxalate. According to Beilstein (1920) the solubility of the sodium salts of the said organic acids decreases in the following sequence: Citrate-tartrate-oxalate. Owing to these conditions of solubility and the slight inhibitory effect of tartrate on complement Wright & Mac Callum (1922) and Quick (1935) later on concluded that the inhibitory effects of citrate and oxalate could not be due to precipitation of Ca.

The conclusion of Wright & Mac Callum and Quick, based on tartrate experiences quite in accordance with those of the writer, seems obvious. However, on comparison between the effects of the said salts on the antibacterial serum processes and the coagulation, respectively, there proves to be close correspondence: *Despite the slight solubility of the Ca-tartrate, sodium tartrate cannot prevent coagulation.* The experiment, Table 33B, shows that, even in concentrations that are 3 or 4 times the citrate concentration required for total inhibition of coagulation, tartrate will only bring about a certain prolongation of the coagulation time. The prolongation corresponds fairly well to the findings after addition of hypertonic NaCl-solution, and on the whole conditions bear a complete resemblance to what was found in the examination of the effect of the same salts on the antibacterial serum activity (Table 33A).—If it is taken for granted that the inhibitory effect on coagulation of citrate and oxalate is due to a Ca-ion fixation, it must be concluded from the above that—despite the slight solubility of the Ca-salt—tartrate does not reduce the Ca-ion concentration so much as do citrate and oxalate,* (the footnote is at the bottom of next page).

The parallelism described between inhibition of coagulation and inhibition of the antibacterial processes can be extended

to the sulphate effect. Already Nissen (1889) described how the alexin process is inhibited by sulphate and his observations were confirmed by Löwit & Schwarz (1903).—The inhibitory effect of sulphate on bactericide was easily demonstrated with the technique employed here. The experiment, Table 35, shows how sodium sulphate concentrations of 0.1 and 0.3 per cent. increase the percentage of survival from 7 to 16 and 27 per cent. respectively.

TABLE 35

Bactericidal Experiment showing the Inhibitory Effect of SO_4 -Ions.

Experimental culture: Breslau 206.

Initial value	Values and degrees of bactericide in fresh serum 35% +		
	saline	Na_2SO_4 0.1%	Na_2SO_4 0.3%
57			
60	8	17	26
67; 61×2	9; 9	22; 20	41; 34
122	7.0 %	16.0 %	27.4 %

As the characteristic great inhibitions of the serum activity have thus proved to be produced by ions combining with Ca, and as the inhibitory conditions correspond entirely to what is known from the coagulation process, *it is natural to explain the effects of citrate and oxalate on the serum activity as a consequence of decreased Ca-ion concentration.* At any rate if, owing to the lacking inhibitory effect of the tartrate on the antibacterial processes, one would discard the hypothesis that citrate, oxalate, and sulphate exercise an inhibitory effect by precipitating Ca, then the universally accepted coagulation theory, according to

* It had been very desirable to compare the inhibitory effects of the said acids with the Ca-ion concentrations in the different reaction milieus. Now, there is a great difference between the reactions of citrate, tartrate, and oxalate with Ca (the first two forming respectively an easily and a less easily soluble complex with Ca, oxalic acid forming a heavily soluble precipitate with Ca), and the writer did not believe to be able to perform the necessary complicated determinations of the Ca-ion concentrations in serum + saline sol. + reagent.

which the inhibitory effects of the same salts on coagulation are due to precipitation of Ca, must be discarded too.

It suggests itself to try to support the Ca-theory by trying to reactivate citrate- and oxalate-blood with Ca. Theoretically a certain reservation must, however, be made against this procedure. As will appear from the inhibition experiments described, a relatively considerable citrate or oxalate concentration is required for the purpose of distinct inhibition; at any rate the concentrations are so large that only part of the citrate or oxalate added may be imagined to combine with Ca. The consequence is that, after addition, the system must be abundant in free citrate or oxalate, and it cannot be precluded in advance that the resulting inhibition is due to free citrate or oxalate and not to combination between these salts and Ca. When now it is tried to reactivate the inhibited serum processes by adding Ca, a double effect is actually produced: The Ca added will not only make up for the loss in free serum Ca caused by the previously added citrate or oxalate, but part of the Ca will combine with citrate or oxalate, thus neutralizing the above mentioned surplus of these salts. A positive result of the reactivation experiment, therefore, is not without ambiguity but actually just means that citrate and oxalate do not irreversibly change the serum.

It was shown by Gengou (1908-1909) that reactivation is actually possible in the case of the hemolysis process, and experiments made by the writer (but not reported here) showed the same as far as the bactericidal process is concerned.

In all the experiments hitherto reported the citrate- and oxalate-inhibitions came out very distinctly and, therefore, it seems peculiar that a number of previous investigators found—mostly on the basis of hemolysis experiments—that citrate and oxalate do not influence the serum activity. This view was advocated in publications by Falloise (1903), Lambotte (1903) and several of their contemporaries and, more recently, in a publication by Ecker & Pillemer (1941). In order to grasp how these investigators arrived at results so entirely the reverse of those of the writer and several others the experimental technique must be considered in detail.

Ecker & Pillemer, whose technique is rather closely described, proceed in the following manner: Equal parts of serum and citrate or oxalate solution are mixed and incubated at 37° for 75 min. At this stage the citrate and oxalate concentrations are so high that, judged by the experiments referred to above, a greatly decreased serum activity would be expected. Before determining the activity Ecker & Pillemer, however, *dilute* in the ratio of about 1:5, and the product treated in that manner is used as complement source for hemolysis experiments. What is of special importance to the comprehension of the results of Ecker & Pillemer and some of the previous investigators is doubtless this dilution that is interposed before the complement activity of the preparation is determined.

Considering the dissociation of the Ca-combinations in the experimental milieu, the following two equations of mass effect must be reckoned with:

$$(1) \frac{C_{Ca^{++}} \times C_{prot. --}}{C_{Ca-prot.}} = K_{Ca-prot.}, \text{ from which the concentration of } Ca^{++} \text{ in serum can be deduced, and}$$

$$(2) \frac{C_{Ca^{++}} \times C_{citr. --}}{C_{Ca-citr.}} = K_{Ca-citr.}, \text{ applying to the dissociation}$$

equilibrium in a solution of Ca-citrate. Lebel (1939) states $K_{Ca-protein}$ and $K_{Ca-citrate}$ to be about 7×10^{-3} and about 6×10^{-4} respectively.

It appears from the above equations that the dissociation degrees of the Ca-combinations increase with the dilution, i.e. on dilution the Ca-ion concentration will decrease less than corresponding to the degree of dilution. As $K_{Ca-citrate}$ is considerably lower than $K_{Ca-protein}$ the citrate concentration will be the quantity of greatest importance to the concentration of Ca^{++} in a mixture of serum and citrate. If the Ca-concentration of normal serum and a citrate concentration necessary to a distinct inhibition be inserted in equation (2) it will be seen that on moderate dilution the concentration of Ca^{++} remains constant or nearly so.

Considering next the proportion between the concentrations of Ca^{++} and complement ($C_{Ca^{++}}/C_{compl.}$) it is obvious that this

proportion will depend on the citrate concentration and decrease when the latter increases. It follows, however, from the dissociation conditions referred to that, if the citrated serum is diluted, $C_{Ca^{++}}/C_{compl.}$ will increase again and approach the initial value; to keep the proportion of the concentrations at a fairly constant level it will suffice, by means of additions from without, to keep the citrate concentration at a constant level in the course of dilution. The writer's own experience is that for the purpose of reducing the serum activity to $\frac{1}{4}$ for instance, about *the same milieu concentration* of citrate or oxalate is required, whether the effect is determined in a phagocytosis experiment with e.g. 55 per cent. fresh serum, or in a hemolysis experiment with less than 1 per cent. fresh serum in the reaction milieu. The hemolysis experiment, Table 36, thus shows that a given oxalate concentration will reduce the complement activity to *the same extent* at different serum concentrations; it will moreover appear from the experiment that at a serum concentration of 0.6 per cent., where there is no excess of complement, an oxalate concentration of 0.25 per cent. is required to discontinue the hemolysis. On comparison with the phagocytosis experiment, Table 33A, where the serum concentration is 55 per

TABLE 36

Hemolysis Experiment with Addition of Na-Oxalate.

Source of complement: Fresh guinea-pig serum. Suspension of blood corpuscles see Table 31.

The concentrations of the normal Na-oxalate are stated in per cent. of the total reaction volume (0.5 cc.). The degrees of hemolysis are stated in per cent. of the total hemolysis.

Milieu-conc. of oxalate	Degrees of hemolysis with the following percentages of complement						
	10	5	2.5	1.25	0.63	0.32	0.16
0.00 %	100	100	100	100	100	70	20
0.07 %	100	100	100	100	90	20	0
0.14 %	100	100	100	100	40	0	0
0.27 %	100	100	90	30	0	0	0
0.54 %	100	80	30	0	0	0	0

cent. but where, at any rate, there is no great excess of serum (pure serum phagocytosis gives 98 per cent. free bacteria), it will be seen that the oxalate concentrations of great inhibitory effect (0.3 and 0.6 per cent.) are of the same magnitude as in the hemolysis experiment. Experience thus seems to indicate that in these experiments it is the proportion $C_{Ca^{++}}/C_{compl.}$ that determines the serum activity.

When it is borne in mind what was stated above, namely that citrate and oxalate do not produce irreparable changes of the serum, it is probable that the effect of dilution described explains how Ecker & Pillemer and others found the same activity in serum and in serum + citrate or oxalate.

Several of the experiments that formed the basis of the investigations into the mode of action of citrate and oxalate are pure phagocytosis experiments and, therefore, the inhibitory effect of the salts has been described as directed against the antibacterial serum activity as a whole. It will be examined in the following whether the inhibitions affect both the alexin and the opsonin processes, or only one of them. For these investigations experiments with preliminary treatment are used as before.

TABLE 37

Experiment with Bacteria preliminarily treated in Fresh Serum and in Fresh Serum + Citrate.

The experiment performed together with that in Table 32. Experimental culture: Breslau 1.

Preliminary treatment	No treatment	Treatment for 30' at 37° in	
		fresh serum 67 % + citrate 1.0 %	fresh serum 67 % + saline
Number of bacteria surviving treatment (initial values) :	219	209	116
	230	219	120
	234; 228	229; 217	124; 120
	100.0 %	95.3 %	52.6 %
Values and degrees of saline phagocytosis :		131	14
	147	137	15
	155; 151	144; 137	25; 18
	66.2 % of 228	63.1 % of 217	15.0 % of 120

In the experiment, Table 37, untreated bacteria are compared with bacteria that have been submitted to preliminary treatment either in fresh serum or in serum + citrate. The experiment shows that a citrate concentration of 1 per cent. discontinues *both* the alexin effect—which gave a percentage of survival of 53—and the opsonin effect—manifesting itself by only 15 per cent. free bacteria among those surviving as compared to 66 per cent. among the untreated bacteria.

The experiment thus shows that—like addition of anticomplementary serum, or NH_3 -treatment of serum—addition of citrate is capable of inhibiting both alexin and opsonin very much.

In the experiments with preliminary treatment it has looked as though the different interferences had discontinued all serum activity; this is, however, hardly so and, as hinted at above, the results should rather be considered high degrees of inhibition. As far as the citrate effect is concerned it has been tried to show that this is so by a special experimental arrangement:

TABLE 38

Examination of the Degree to which the Bactericidal Effect is inhibited by Citrate in High Concentration.

In order to disclose possible small variations in the bacterial density within the period of inoculation determinations were made twice at an interval of 15-20 min.

Experimental culture: Breslau 1.

Time of withdrawal for inoculation	Experimental milieu			
	saline	heated serum 55%	heated serum 55% + citrate 1.2%	active serum 55% + citrate 1.2%
0 min.	481 510; 496		479	450
		460	485	451
		498	514	470
		500; 486	524; 501	511; 471
		492	459	452
about 18 min.		498	498	471
		512; 501	508; 488	478; 467
Averages:	496	493	495	469.0
		494.5 = 100 %		= 94.9 %

Comparison of the values 494.5 and 469 gives $t = 2.30$ (cf. text on p. 64). The P-values 83 % and 52 % are found corresponding to the values 494.5 and 469 respectively.

In the bactericidal experiment, Table 38, several controls are included; the initial value was thus determined not only in saline solution but also in inactive serum and inactive serum + citrate, and withdrawals were made twice from each experimental test tube at suitable intervals to disclose possible alterations of the number of bacteria in the course of inoculation. The initial value fixed in this manner on the basis of 15 single determinations is compared with the bactericidal value determined through 7 single counts from a test tube with fresh serum + citrate. The comparison shows that even the high citrate concentration employed (1.2 per cent.) does not completely efface the alexin effect; it can be established fairly safely that the difference between initial value and bactericidal value is about 5 per cent. ($t = 2.30$).—This experiment with the many single counts for the purpose of determining the same value also illustrates the possibilities and limitations of the counting technique in an excellent manner; cf. the record of the experiment under reference to figures, pages 64-65.

The results of the qualitative investigations can be summarized as follows:

The difference between alexin and opsonin described by Gordon and collaborators and said to be that the fourth component is necessary to the alexin process and not to the opsonin process was not found by the writer. On the contrary, the experiments seem to indicate that the fourth component is necessary to both processes (Chapter VII).

As referred to in the introduction, there was every reason to consider alexin and opsonin identical until Gordon's investigations were published. After the investigations reported in Chapter VII it therefore seems natural to revert to the old perception as a hypothesis.

The experiments with anticomplementary serum and with citrate and oxalate (Chapters VIII and IX) were performed to support the "identity hypothesis". None of these substances have been previously employed in direct comparisons between alexin and opsonin, but it was known in advance that they influenced at any rate one of the antibacterial functions.—The experiments

in Chapters VIII and IX showed that both the alexin and the opsonin processes are greatly inhibited on addition of anticomplementary serum or citrate; in the case of citrate (and oxalate) it was moreover shown that, probably, the inhibitory effect is due to a certain amount of free Ca being necessary to the functions of alexin and opsonin. As hinted at in the introduction the writer, therefore, believes that Ca should be considered an "alexin- and opsonin-component."

On the basis of the qualitative investigations there seems altogether to be good reason to maintain the identity hypothesis and, while looking for further confirmation, to consider alexin and opsonin identical.

CHAPTER X

TITRATION EXPERIMENTS

In the references to the single experiments with preliminary treatment in Chapters VII to IX it was emphasized that, although the inhibitions often seem to be absolute, they are presumably only particularly high degrees of inhibition; this means that the qualitative investigations have been directed towards a few extreme points of the different courses of inhibition. It will be natural, therefore, to broaden the knowledge of the alexin and opsonin processes by supplementing the qualitative experiments with investigations covering the entire range of reactions, right from the natural uninhibited alexin and opsonin effects to the greatly inhibited, almost discontinued effects observed in high concentrations of for instance anticomplementary serum or citrate.

The form of experiment that may be considered here has already been used in some cases—especially in bactericidal investigations. In Chapter II (Table 1) an examination of the range of reaction of the alexin was reproduced, performed as a “dilution titration”; and in Part II the effect of the antibodies on the alexin process was examined over wide ranges by means of titrations in which the concentrations of antibodies varied. In the following discussions it should be borne in mind that the shape of curve obtained when the course of the bactericidal process is titrated, e.g. by means of dilution of the active serum, gives a picture of the distribution of the bacteria according to alexin sensitivity (cf. Chapter II, page 27).

When “corresponding bactericidal and phagocytosis curves” are mentioned in the following, this means titration curves of experiments in which the alexin effect and the total serum ac-

tivity are examined *simultaneously* over a wide range. The examinations are made either by means of gradual dilution of the active serum and termed "dilution experiments", or by means of addition of gradually increasing amounts of inhibitory substance, the latter experiments being termed "inhibition experiments" or "inhibition titrations."

Before embarking on comparisons between alexin and opsonin titrations it will be necessary more closely to examine what might be expected theoretically from such comparisons. It is, of course, desired especially to get to know if titration experiments can be used for the purpose of testing the postulate of the identity hypothesis: That alexin and opsonin are identical and that, actually, we thus have to do with only one active substance, the effect of which on the bacteria can just be observed in two different ways; partly by means of the stimulation of phagocytosis occurring at a rather early stage and partly by means of the subsequent bactericide.

Although the experiments employed to record phagocytosis and bactericide, respectively, are of rather a special nature, the problems associated with the investigations are of a universal character and resemble in detail the problems of certain animal experiments: If for instance, for the purpose of comparison, we take an experiment with injection of tetanus toxin into mice, the early degree of influence, manifesting itself in the bacteria by increased phagocytability, can be compared to the muscular rigidity that is the first symptom in the mice, while the bactericide corresponds direct to the deaths of the experimental animals. For the sake of comparability the experiment on mice should be imagined to be arranged in the manner of the bacterial titration experiment, i.e. that different doses of toxin would have to be administered each to its very large group of animals.—When now a suitable time for reading the experiment is chosen, and if the number of distinctly rigid mice as well as the number of dead mice are recorded within each group of animals, a material will be obtained from which the distribution as to resistance to tetanus toxin of the population of mice can be determined in two ways, i.e. from one or the other of the criteria used: "Rigidity" or "death". The two distributions may be iden-

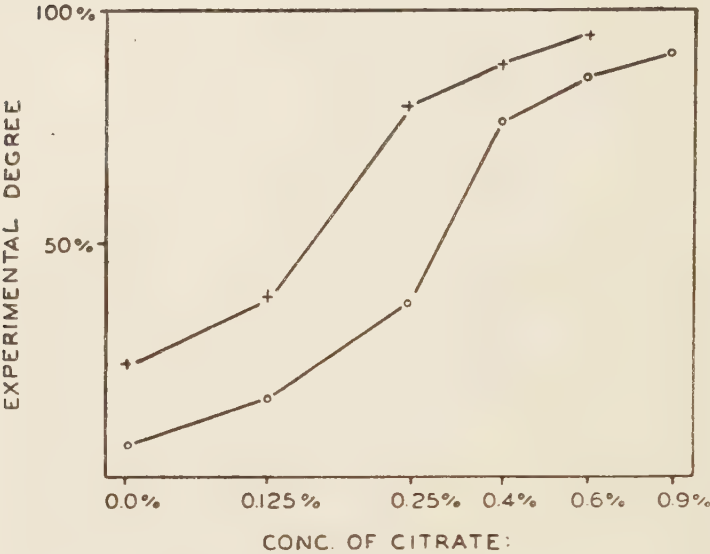
tical, which will imply that the ratio between the rigidity-producing and the lethal dose of toxin is constant in all animals, or there may be a greater or less difference between the distributions. What is of greatest interest here is that as long as we are dealing with different effects of *one* toxin, *the same ratio between the two distributions must be expected, whether the toxin be diluted or the amount of free toxin is reduced in some other way*, e.g. by adding increasing doses of a non-toxic adsorbent. If, on the other hand, the toxin preparation is imagined to contain *two* fractions producing rigidity and death, respectively, in the mice, it is no longer probable that the same relation will always be found between the two distributions. An adsorbent which in full doses will adsorb both toxin components greatly may for instance well be more active against one component than against the other, and the curves obtained in experiments with portions of toxin that are adsorbed to a gradually increasing extent will then often be placed in relation to each other in a manner quite different from the curves of an experiment in which the toxin preparation is diluted.

When these general considerations are conveyed to the bacterial experiments, the following reasoning is arrived at: If it can be ensured that the agents used for the purpose of inhibiting the alexin and opsonin processes are not at the same time bacterial or leukocytic poisons, the following requirement must be made, provided the identity hypothesis has to be maintained: *The mutual relation between corresponding bactericidal and phagocytosis curves must be independent of the titrations being performed as dilution or inhibition titrations.*—On the other hand, if the identity hypothesis is not tenable, certain differences between alexin and opsonin, which cannot be demonstrated in qualitative examinations, must be expected to manifest themselves in the manner that, in one or more inhibition experiments, titration curves are found that are placed differently in relation to one another than might be expected from a simple dilution experiment.

In order to show the relations generally found between corresponding bactericidal and phagocytosis curves, two citrate ex-

TABLE 39

Titration of the Bactericidal and Phagocytosis Inhibitions caused by Citrate.



Upper curve: Bactericidal degrees, marked with +.
Lower curve: Degrees of total serum activity, marked with o.
Serum conc.: 55 %. Experimental culture: Breslau O-form.
The immobile O-form contains no H-antigen and, as shown by the figures below, no detectable phagocytosis occurs in saline milieu:

	220
Initial value determined by inoculations from the control tube	230
during the experimental period	236; 229
	204
Initial value determined from a tube incubated for 30 min.	208
at 37°	220; 211
	210
Saline phagocytosis value determined after incubation for	211
30 min. at 37°	242; 221

In all three cases the same degree of centrifugation was applied; comparison of the values 229, 211 and 221 gives $P = 68 \%$.

periments are reported below, which besides tend to illustrate certain special conditions:

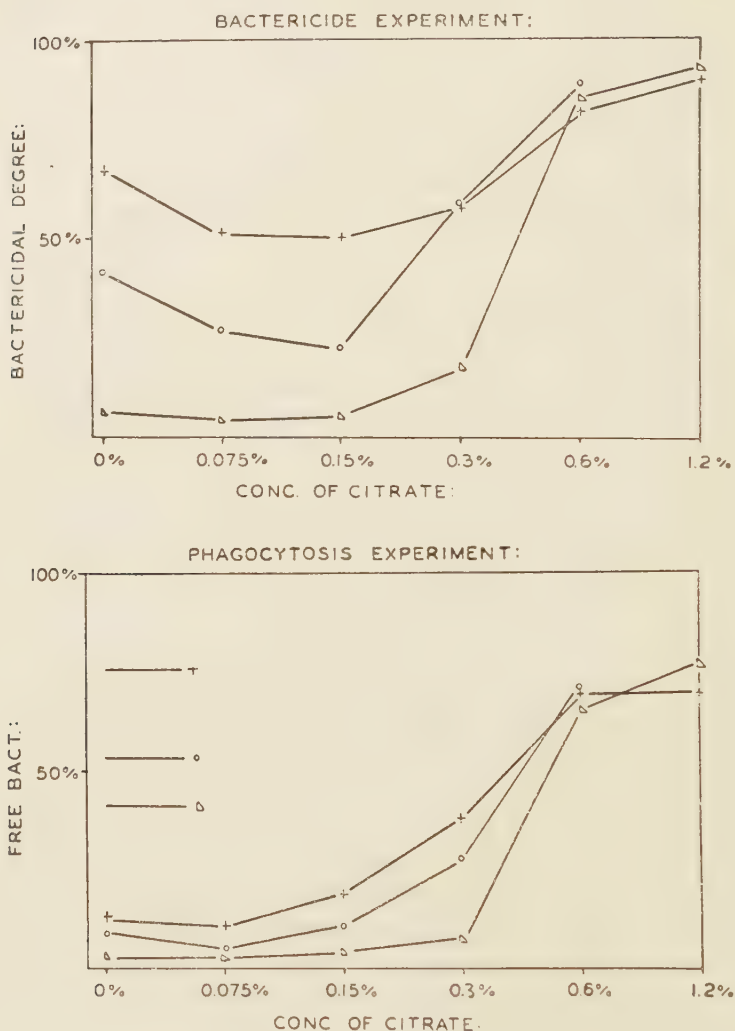
The experiment, Table 39, was performed with the Breslau-O-form referred to earlier, and it will be seen how the curves take a fairly parallel course from bactericidal and phagocytosis degrees of about 25 per cent. and about 10 per cent., respectively, to values of 90 to 100 per cent. when the citrate concentration increases. As pointed out earlier the O-form is peculiar in that it is non-phagocytable in inactive milieu; this special property appears from the control determinations of saline-phagocytosis shown in Table 39.

The experiment, Table 40, shows similar citrate titrations made simultaneously with three Breslau strains of different sensitivity—Nos. 1, 50, and 206. As in the experiment with the O-form it will be seen that corresponding bactericidal and phagocytosis curves take a fairly parallel course, but at the same time it is seen that in these cases the phagocytosis curves do not approach 100 per cent.-values but end in a common zone of 70 to 80 per cent. free bacteria. It appears from the Figure that in the case of Breslau 50 and 206 there is a considerable difference between the end values of the phagocytosis curves and the saline-phagocytosis values of the same strains; as referred to on page 76, this must be considered a consequence of the fact that the inhibitory effect of the thermostable serum substances on phagocytosis asserts itself in the greatly inhibited milieu.

The latter experiment is moreover peculiar in that in all the three strains the weakest citrate concentrations *increase* the serum activity. A similar effect was described by Pettersson (1902) as the sole effect of citrate and oxalate additions to serum in bactericidal experiments. When the characteristic inhibitory effect was not observed by Pettersson it is doubtless due to the fact that he used citrate and oxalate plasma that was diluted before use. The paradoxical effect of citrate appears irregularly and is apparently associated with certain serum portions. It is possible that the phenomenon may be connected with the Ca-concentration or the conditions of solubility of Ca in the sera in question (cf. pages 127-129). It is known from the writer's experiments that an excess of Ca exercises a moderately inhibitory effect on the

TABLE 40

Titration of the Bactericidal and Phagocytosis Inhibition caused by Citrate.



Serum conc. in all tubes 55 %; the phagocytosis experiment carried out the day after the bactericidal experiment.

Experimental cultures: Breslau 1, 50 and 206, marked with +, o and Δ respectively.

The degrees of saline-phagocytosis stated by the horizontal lines accompanied by the signature of the strain in question.

serum activity and it is known about the coagulation process that there is an optimal Ca-concentration at which the process is most rapidly completed. By way of explanation of the observations made in the experiment, Table 40, it might therefore be imagined that in certain serum portions and at certain dilutions the Ca-ion concentration will only reach values that are optimal to the alexin process after addition of small amounts of citrate.

We have seen from the above citrate experiments that corresponding bactericidal and phagocytosis curves often take a fairly parallel course. It suggests itself at first glance that this must be understood so that the distribution of the bacteria according to alexin and opsonin sensitivity is almost the same. It is, however, necessary to make certain reservations in the estimation of comparative experiments comprising both bactericidal and phagocytosis titrations. While in the former titrations the results of the experiments are obtained direct as percentages of survival—i.e. on immediate observation of the state of the bacteria—we must be content in phagocytosis experiments to record the state of the bacteria—in this case their phagocytability—by means of the reaction of the leukocytes to the bacteria in question. In the latter case it will, therefore, be necessary to deliberate whether the mutually different capacity for phagocytosis of the leukocytes and the chances of interference between leukocytes and bacteria will influence the shape of the phagocytosis titration curves.

This problem is fairly perspicuous if it is imagined it is possible to follow the fate of an average bacterium. In an experiment with very intense phagocytosis this individual bacterium will have nearly a 100 per cent. chance of being phagocytized—i.e. of meeting a leukocyte with so great a capacity for phagocytosis that the bacterium is phagocytized. Supposing now the serum concentration and with that the opsonin activity are reduced by means of dilution, the phagocytability of the bacterium will decrease simultaneously, and corresponding to each phase of a series of dilutions the individual bacterium will thus have a certain phagocytability. The result of decreasing serum concentration will therefore be that a constantly decreasing number of

the leukocytes present will be able to phagocytize the bacterium, whose chances of being phagocytized will thus decrease. In other words: In its effect on the degree of phagocytosis, dilution of the serum corresponds to gradual "inactivation" of the leukocytes, those with the slightest capacity for phagocytosis being affected first.

If, for the sake of simplicity, a milieu is imagined in which at a given opsonin activity all leukocytes are active and the serum is then diluted step by step according to the scale used in bactericidal experiments, the leukocyte population will be split up in groups of varying size as the leukocytes are gradually "inactivated." First, small groups with the poorly phagocytizing leukocytes, next larger groups with medium phagocytizing leukocytes and lastly, small groups with highly phagocytizing leukocytes.—The distribution of leukocytes according to capacity for phagocytosis was examined by Hald, Jersild & Rasch (1943); based on Jersild's experiments it was found that the distribution of phagocytized bacteria in a certain number of leukocytes was of normal type (in experiments with slight phagocytosis only curtailed normal distributions were found). As was pointed out by Hald, Jersild & Rasch, the distributions observed are hardly determined solely by the mutually different capacity for phagocytosis of the leukocytes but should be viewed as a resultant of the distributions of the leukocytes and bacteria according to capacity for phagocytosis and phagocytability respectively. When the distribution observed is normal it is most natural to suppose that both of the distributions concerned are normal too.—The phagocytability of the bacteria is a function of their opsonin sensitivity; now that it is known (cf. page 144) that the distribution of the bacteria according to alexin sensitivity is normal and will manifest itself thus when the serum is diluted step by step according to a logarithmic scale it will be reasonable to suppose as a hypothesis that the distribution of the bacteria according to opsonin sensitivity is normal, too, and can be elicited similarly. The latter supposition means that in the gradual "inactivation" of the leukocytes described above they will be split up in groups corresponding to the supposed normal distribution according to capacity for phagocytosis.

It is, of course, difficult to determine in advance the number of the leukocytes present that will be actively phagocytizing at a given serum concentration, and it is just as difficult to determine the number of leukocytes "inactivated" at a given decline of the serum concentration. If, however, the curve of Table 5 is considered, showing the dependence between leukocyte figure and degree of phagocytosis, it is possible to form an impression of how the shape of this curve of Table 5 will influence the shape of the phagocytosis curve in a dilution experiment:

Let us first arbitrarily divide the abscissa in Table 5 in such a manner that the size of the groups of blood corpuscles into which it is divided will correspond to a larger or smaller section of a normal distribution. If it is now taken for granted that one of the leukocyte groups is "inactivated" for each of the dilution steps of the titrations—i.e. that the leukocytes are normally distributed according to capacity for phagocytosis—the phagocytosis degrees corresponding to the opsonin activity at the dilution degrees in question can be read from Table 5. If it is considered that the phagocytosis degrees in Table 5 are computed as percentages of the bactericidal value, it will be possible by means of an ordinary bactericidal curve (showing the normal distribution of the bacteria according to alexin sensitivity) to convert the phagocytosis degrees read from Table 5 into degrees of total serum activity. In this manner a theoretically possible phagocytosis curve is arrived at, corresponding to the bactericidal curve employed, when the leukocyte groups selected are of a suitable size. Computations of this nature have shown that if it is taken for granted that the leukocytes are normally distributed according to capacity for phagocytosis, theoretical phagocytosis curves differing somewhat in shape from the corresponding bactericidal curves will be arrived at, owing to the shape of the curve in Table 5. If transformed by means of probits, the ordinary bactericidal curves thus being transformed into straight lines, (cf. page 144) the theoretical phagocytosis curves appear to differ from the straight line, as they display a more or less marked curve resembling the one seen in the experimental phagocytosis curves in Tables 41B and 43A.

This rough estimate of the importance of the leukocyte dis-

tribution and the chances of interference between leukocytes and bacteria to the shape of the phagocytosis curves thus leads to the following reasoning: When the distribution of the leukocytes according to capacity for phagocytosis is allowed to follow an arbitrary normal distribution, phagocytosis curves differing in shape from the corresponding bactericidal curves will be arrived at when the dependence between leukocyte figure and phagocytosis degree is of the type reproduced in Table 5. It will not be attempted to make a more thorough examination of the quantitative alterations which the phagocytosis curves must be supposed to pass through owing to the conditions referred to; it will suffice to point out that changes of the phagocytability of the bacteria as a consequence of serum dilution or inhibition of the opsonin activity are not the only factors to determine the shape of the phagocytosis curves. *Therefore a phagocytosis titration curve cannot be expected to give an exact picture of the distribution of the bacteria according to opsonin sensitivity.*

As a consequence of the above considerations the experiments that will be described now were arranged in the following manner: Each complete experiment requires a comparison or "scale" experiment consisting of a bactericidal and a phagocytosis curve obtained by means of dilution titration; at the same time a similar pair of curves is determined by means of titration of one of the inhibitory effects referred to earlier, and the purpose of the experiment is to examine whether there is any difference between the mutual situation of the curves, according to their being obtained by means of dilution- or inhibition-titration. It was shown earlier that on the basis of the identity hypothesis it must be required that the mutual situation of the curves is the same, irrespective of the manner in which the titration was performed, provided only that bacterial or leukocytic poisons are not introduced in the course of the experiment. The following titrations will show how strictly this requirement is complied with in comparisons between dilution titrations and titrations of the citrate effect, the effect of anticomplementary serum, and that of NH_3 -treatment respectively.

QUANTITATIVE CITRATE EXPERIMENTS

Certain special conditions must be mentioned to begin with; one applying especially to the citrate experiments, and some applying to the combined titration experiments as a whole.

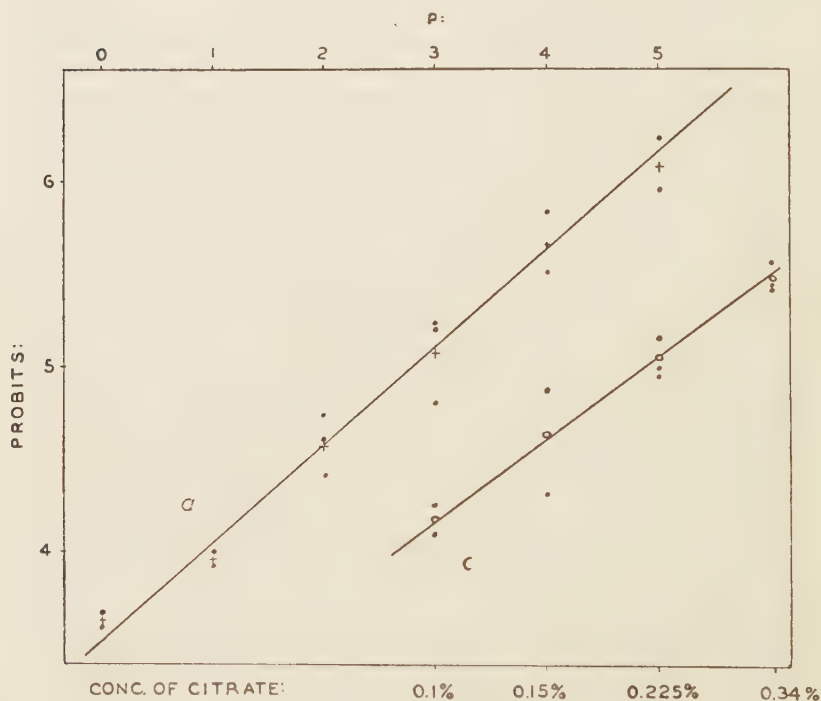
First, it must be pointed out that in the concentrations employed citrate does not injure bacteria or leukocytes: On page 123 a control experiment was reported, showing that the effect of citrate on the osmotic pressure is without any importance to phagocytosis, and a series of other control examinations in connection with different experiments showed that citrate does not influence the spontaneous phagocytosis.

It should be borne in mind that it is a characteristic feature of the titration experiments as a whole that, owing to the influence of the thermostable serum substances on the bactericidal and phagocytosis processes, it is necessary to keep the total serum concentration at a constant level in all experimental test tubes. As far as the dilution titrations are concerned this is done by diluting the active serum in inactive serum.—Moreover it should be borne in mind that, owing to the spontaneous phagocytosis remaining even after discontinuation of all serum activity, a special basal value for the computation of the phagocytosis degrees has been introduced. The new value is equal to the phagocytosis value in a control test tube containing purely inactive serum and, as referred to earlier, it represents the natural limit of the phagocytosis titration.

In the graphic reproduction of the experiments a special procedure was used in several cases, the bactericidal and phagocytosis degrees being expressed, not in per cent., but by means of "probits". The passing from per cent. to probits is effected by means of Fischer & Yates's table (1938) in which the probit corresponding to a given percentage is looked up. This table is computed in the manner that if a curve—e.g. the bactericidal curve in Table 1—is the outcome of "normal" distribution of the bacteria as to alexin sensitivity, the S-shaped curve will be transformed to a straight line when passing from per cent. to probits. The appearance of the straight line is thus the test that the original curve represents a normal distribution; the reci-

procal value of the slope of the straight line will be equal to the standard deviation of the normal distribution in question. As most bactericidal curves proved to be transformable into straight lines by means of probits, this must be considered an indication of the fact that most frequently the individuals of a bacterial population are normally distributed according to alexin sensitivity. The resistance distribution against alexin is thus of the same type as that against different disinfectants (Ipsen, 1941). The

TABLE 41A
Bactericidal Titrations on Dilution (a) and on Addition of Citrate (c).



Mean values in the curves a and c marked with + and o respectively; single determinations marked with dots.

Total conc. of serum (active + inactive) the same in all tubes = 35 %.

Conc. of active serum: 35(0.67)^P %.

Initial value: 166. Experimental culture: Breslau 206.

TABLE 41B

Phagocytosis Titrations on Dilution (b) and on Addition of Citrate (d).



The curves b and d marked with + and o respectively; single determinations marked with dots.

Total conc. of serum and conc. of active serum as in Table 41A.

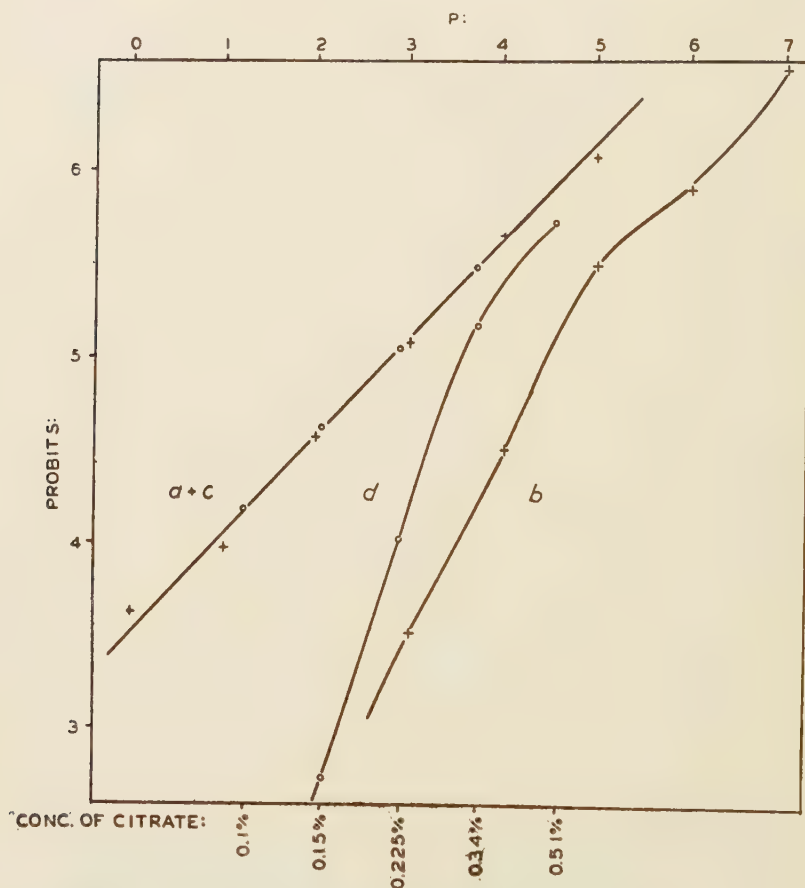
The degrees of phagocytosis, or more correctly the degrees of total serum activity, are calculated as described on p. 143. Basal value, (i.e. the phagocytosis value in milieu with 35 % inactive serum): 135.

The titrations a, b, c and d made at the same time and with the same culture.

fact that one has to do with normal distributions means again, of course, that in practice the employment of probits is very advantageous, as the simplest possible shape of curve is obtained by this means.

The bactericidal curves of simultaneously performed dilution

TABLE 42
Joint Representation of the Curves a, b, c and d from Tables 41A and B.



Mean values from the dilution titrations marked with +.

" " " " titrations with citrate marked with o.

The conc. of citrate marked out on such a scale that the linear curves a and c coincide. For experimental details see Tables 41A and B.

and citrate titrations are reproduced in Table 41A (curves a and c). As will appear from the table, two straight and almost parallel bactericidal curves are obtained when the same dilution phases are used in the serum and the citrate series and with logarithmic dilution scales. In other words: The individuals of the bacterial culture employed are approximately normally distributed according to alexin sensitivity, *and* the effect of a certain rise of the citrate concentration corresponds very closely to the effect of an equally great decline of the concentration of active serum. The alexin activity is thus inversely proportional to the citrate concentration, or nearly so; a relation corresponding well to the one that is found between complement activity and the oxalate concentration, as will appear from the hemolysis experiment, Table 36.

Table 41B reproduces the phagocytosis curves b and d of the dilution and the citrate titration respectively. These curves resemble each other closely but differ from the straight lines in Table 41A, which appears distinctly from the two treble determinations, one in each curve, falling entirely outside the connecting line between the terminal points of the curve in question. As to the shape of the phagocytosis curves, see also the theoretical discussion on pages 139-142.

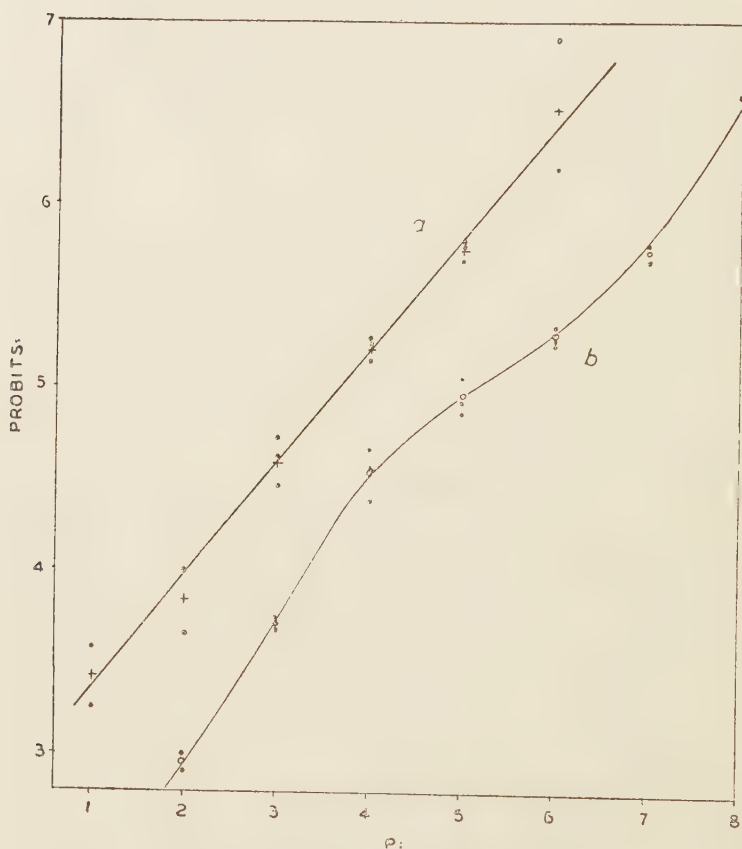
Table 42 reproduces all the four titration curves of the citrate experiment as a whole, the bactericidal curves a and c having been made coinciding by means of a slight alteration of the scale of the inhibition experiment. According to the considerations gone through on page 135, the identity hypothesis now requires that the two phagocytosis curves will also coincide, which, however, proves not to be the case. As will appear from the table, the phagocytosis curve (d) of the inhibition experiment separates to an increasing extent from curve (b) of the dilution experiment, approaching the bactericidal curves (a + c) as the citrate concentration gradually increases.—The importance of this deviation, which recurs in the following inhibition experiments, will be discussed in detail in the next chapter.

QUANTITATIVE EXPERIMENTS BY USE OF ANTICOMPLEMENTARY SERUM

It appeared in a number of control experiments that anti-complementary heated serum influences the spontaneous phago-

TABLE 43A

Titration of Bactericide (a) and Phagocytosis (b) on Dilution.



The curves a and b marked with + and o respectively; single determinations marked with dots.

Total conc. of serum: 55 %. Conc. of active serum: 17.5(0.6)P %.

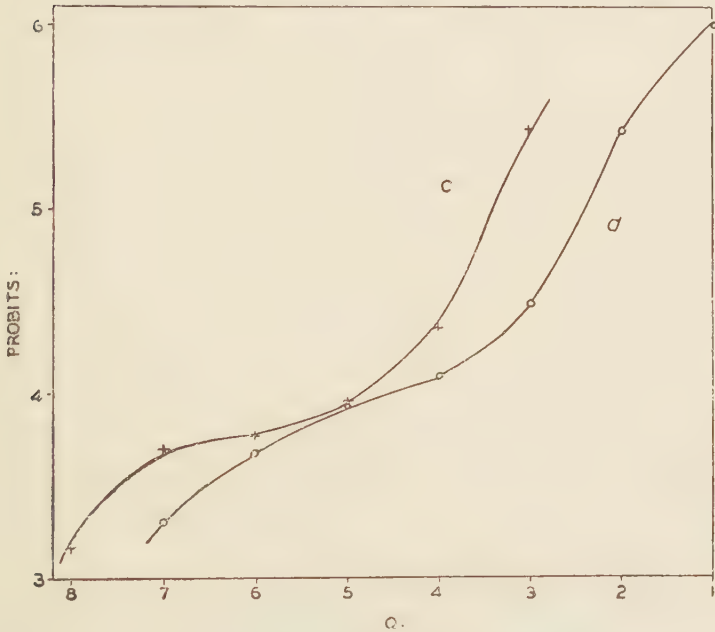
Initial value: 254. Basal value for the phagocytosis experiment: 226, (cf. p. 143). Experimental culture: Breslau 206A.

cytosis in exactly the same manner as normal heated serum. There is thus no reason to believe that, in the form in which it is employed in the experiments, anticomplementary serum exercises a harmful effect on bacteria or leukocytes.

The first titration experiments with anticomplementary serum showed that the inhibition curve had a peculiar shape and followed a very steep course. This fact necessitated the employment of a particularly sensitive bacterial strain and small intervals between the concentrations of anticomplementary serum. The strain

TABLE 43B

Titration of Bactericide (c) and Phagocytosis (d) on Addition of Anticomplementary Serum.



The curves c and d marked with + and o respectively; single determinations not included in the figure.

Total conc. of serum: 55 %. Conc. of anticomplementary serum: 2(0.75)Q %, (the anticomplementary serum diluted in inactive serum).

Initial and basal values and experimental culture as in Table 43A.

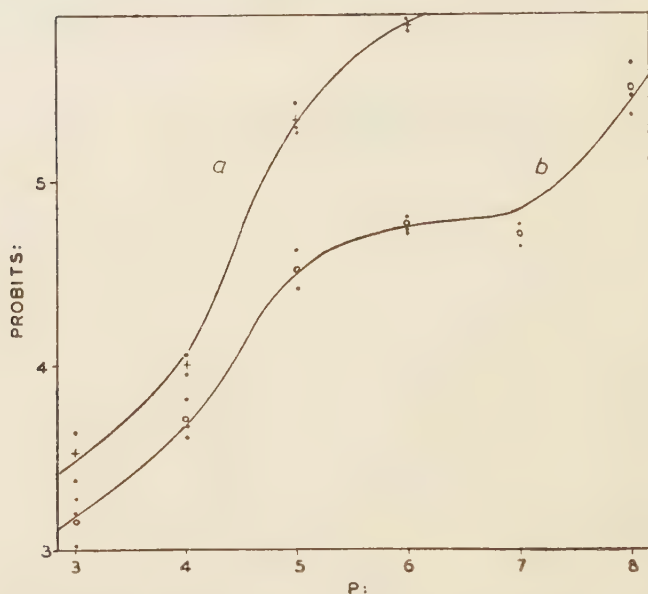
The mean values +5, +6, +7, o3 and o5 determined by three counts each, the rest of the values are from duplicate determinations.

employed is termed Breslau 206 A; it was made from Breslau 206, which was rendered further sensitive by means of a series of passages in bouillon at 37°.

Two titrations in connection with which dilution titrations

TABLE 44A

Titration of Bactericide (a) and Phagocytosis (b) on Dilution.



The curves a and b marked with + and o respectively; single determinations marked with dots.

Total conc. of serum: 55 %. Conc. of active serum: 35(0.6)^P %.

Initial value: 328. Basal value for the phagocytosis experiment: 252, (cf. p. 143).

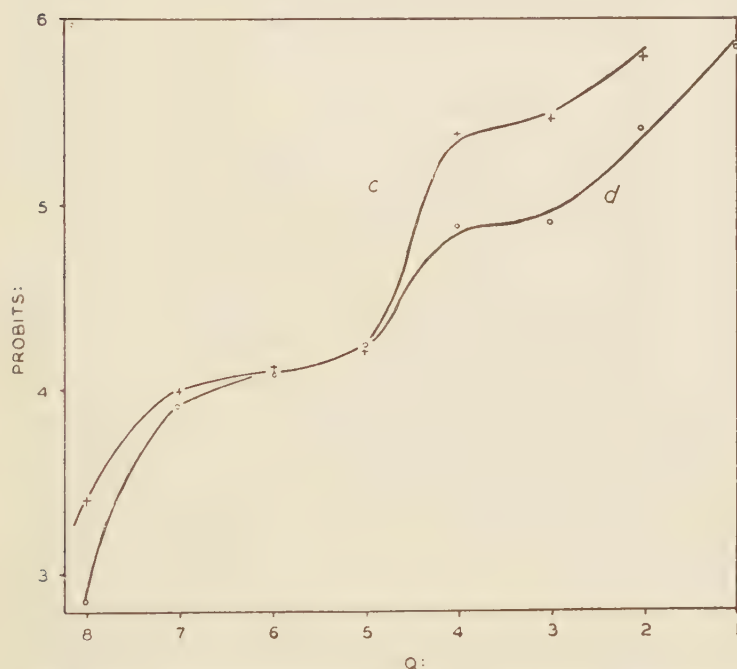
Experimental culture: Breslau 206A.

In order to arrive at the best possible comparability between the curves a, b, c and d a special technique has been employed in this and the following titrations: All test tubes were shaken gently immediately after the bacterial suspension was added and the addition of bacteria and the interruption of the experiment were carried out in such a way that tubes from the different titrations, known to give values in the same interval, were treated in sequence. This means that slight differences in the experimental time between tubes treated early and tubes treated late will influence the four curves in the same way.

were made will be reported here out of a number of titrations which displayed the same fundamental conditions all of them. The curves are termed as in the above citrate experiment: a and b being the bactericidal and phagocytosis curves of the dilution experiment, c and d the corresponding curves of the inhibition experiment. Owing to the shapes of the c- and d-curves there is no object in reproducing the bactericidal and phagocytosis curves separately as in the citrate experiment, in which the a,

TABLE 44B

Titration of Bactericide (c) and Phagocytosis (d) on Addition of Anticomplementary Serum.



The curves c and d marked with + and o respectively; single determinations not included in the figure.

Total conc. of serum: 55 %. Conc. of anticomplementary serum: $2(0.75)Q$ %, (the anticomplementary serum diluted in inactive serum).

Initial and basal values and experimental culture as in Table 44A.

The mean values +3, +5, +7, o3, o5 and o7 determined by three counts each, the rest of the values are from duplicate determinations.

c- and b, d-curves in pairs resembled each other closely. In the following experiments the dilution and inhibition experiments will, therefore, be reproduced separately.

Table 43A represents a typical dilution experiment: As in the citrate experiment the bactericidal curve (a) forms a straight line in a probit diagram (it is the same curve as is reproduced in Table 1 in its original S-shape, as it appears before the transformation). The phagocytosis curve (b) deviates from the straight line almost in the same manner as in the citrate experiment, but runs largely parallel to the bactericidal curve. Table 43B represents the curves of an inhibition experiment performed simultaneously. The bactericidal and phagocytosis curves (c and d) resemble each other much but are of a peculiar shape with a steep rise interrupted by a plateau formation at about the middle. Besides the characteristic shape the mutual situation of the inhibition curves is, however, somewhat striking; as in the citrate experiment the phagocytosis curve (d) of the inhibition experiment is situated closer to the corresponding bactericidal curve (c) than might be expected after the dilution experiment. This is distinctly seen if, by means of the curves a, b, and c and on the basis of the identity hypothesis, the d-curve is to be constructed. In case the dilution experiment is used as relation diagram it should be possible according to the identity hypothesis direct to read the phagocytosis degrees corresponding to the bactericidal degrees of the inhibition experiment; it will be seen at first glance from Tables 43A and B that a d-curve constructed in this manner, though starting at the same value, will lie considerably below the experimental d-curve.

Tables 44A and B represent another titration experiment that has been included, not only because it confirms the characteristic findings referred to above, but also because it displays a peculiarity that has been observed only this once. With the exception of the bactericidal curve of the dilution experiment, which has not been carried through so far as the other curves, all the titrations display plateau formation in *two* places; besides the low plateau, which was found in all titrations with anticomplementary serum, an additional plateau is found at a considerably higher level. The explanation of this peculiar fact

is most likely that, for reasons which it has been impossible to account for after the experiment, a bacterial population comprising two groups of different sensitivity has been involved, one of them having been slightly less, the other slightly more sensitive than the homogeneous culture that was used in the experiment, Table 43. The fact that the additional plateau formation is found both in the bactericidal curve that has been carried on sufficiently far and in both phagocytosis curves suggests that the sensitivity of the bacteria to alexin and that to opsonin keep abreast fairly well.

As referred to, the latter experiment displays the same characteristics as the former. In both experiments the inhibition curves are closer to one another than might be expected according to the identity hypothesis.

QUANTITATIVE EXPERIMENT WITH AMMONIA-TREATED SERUM

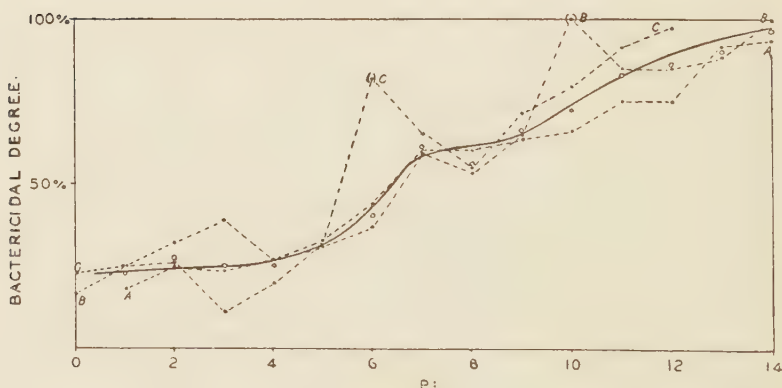
In principle the effect of NH_3 -treatment can be titrated in two different ways. A number of serum portions treated with different intensity can be used or, instead of titrating the effect of destruction in this manner, the effect of the reactivation process may be titrated by adding increasing amounts of heated serum to serum that has been completely inactivated by ammonia. The latter procedure was preferred, for besides giving information about the effect of partial removal of the fourth component it makes it possible to examine whether there is an excess or a deficiency of the fourth component in normal serum. As referred to on page 104, the titration of the reactivation process has shown that usually there is a great excess of the fourth component in normal serum.

Several control experiments showed that ammonia-treated serum and heated serum influenced the spontaneous phagocytosis alike; it is therefore reasonable to presume that neither bacterial nor leukocytic toxins are added or produced in the course of the treatment of serum with ammonia.

For certain reasons the titration experiments that will be de-

scribed now are not so complete as those referred to earlier in this chapter. It has proved that, even if test tubes that have been especially thoroughly cleaned are used, the reactivation fails more or less completely now and then in an otherwise continuous titration series. This is presumably due to the fact that impurities in test tubes or pipettes may destroy the very small amounts of the fourth component involved.—These difficulties in connection with the peculiar shape of the titration curves made it necessary to make numerous and closely juxtaposed determinations, and the reactivation experiments had thus alone reached an extent that made it impossible to combine them with the ordinary dilution experiments. In order to make up for this shortcoming the ammonia experiments were performed with the strain Breslau 206, which is well-known from many previous titrations, and a

TABLE 45
Three Bactericidal Experiments with Titration of the Reactivation of NH_3 -Serum.



Besides the three individual curves (mean values marked with dots) the figure includes an average curve marked with o, cf. text on p. 155.

Curve A: Initial value 265.

„ B: „ „ 170.

„ C: „ „ 172.

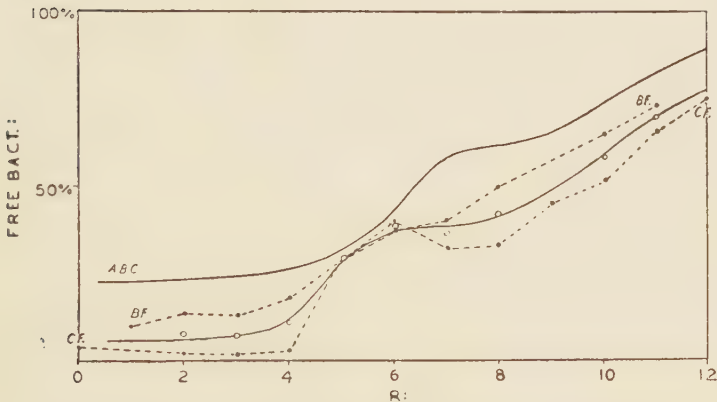
Conc. of NH_3 -serum: 50 % (corresponding to 33 % undiluted serum).
Reactivation dose of inactive serum: 10(0.5)^R %.

Experimental culture: Breslau 206.

series of identical reactivation titrations was made; despite the difficulties it is thus possible to get a reliable impression not only of the shape of the curves but also of their mutual relation.

In Table 45 a group of three uniform bactericidal titrations is reproduced (curves A, B, and C). As will be seen the curves agree fairly well, and if the two cases of failing reactivation (one in each of the curves B and C) are excluded it is possible to draw a representative mean curve. The shape of this curve is extended and has a plateau formation reminding of the one found in the experiments with anticomplementary serum. As pointed out above, the reactivation curve also shows that only very small amounts of the fourth component are required for partial reactivation. As none of the curves can be reduced to a simpler form by means of probits, the ammonia titrations are

TABLE 46
Two Phagocytosis Experiments with Titration of the Reactivation of NH_3 -Serum.



As in Table 45 the figure includes an average curve (marked with o) and individual curves marked with dots. For the purpose of comparison the average curve (ABC) of Table 45 is drawn up.

Curve BF: From a titration made together with B of Table 45.

" CF: " " " " " " " C " " 45.

For experimental details see Table 45.

Experimental culture: Breslau 206.

reproduced in the ordinary way with the survival percentage as ordinate.

Table 46 represents two phagocytosis titrations carried out simultaneously with the titrations B and C in Table 45. The phagocytosis curves, too, agree fairly well, and it is thus possible to draw a mean curve representing the two experiments. For the purpose of comparison with the bactericidal experiments the mean curve of the latter, ABC, has been transferred to Table 46. As in the previous titrations it is found here that the bactericidal and phagocytosis curves agree in shape as well as in extension. Considering the experience from several dilution experiments with the same strain (Breslau 206) it can be said with great certainty that the two mean curves of the reactivation experiments are situated closer to one another than might be expected from the identity theory.

The results of the combined titration experiments can then be summarized as follows: As regards shape and extension all experiments show good agreement between corresponding bactericidal and phagocytosis curves. As to the relation between the curves it appeared that, in all inhibition and reactivation experiments, corresponding curves lie closer to one another than might be expected from the identity theory and the dilution experiments. The implications of that observation will be discussed in the next chapter.

CHAPTER XI

DISCUSSION OF THE TITRATION EXPERIMENTS

It is very remarkable that, as regards the relationship between the curves, *all* the inhibition and reactivation titrations dealt with in the preceding chapter display the *same* deviation from what might be expected from the identity theory and the dilution experiments. At first glance it appears more probable that these uniform deviations are due to some hitherto unnoticed property of the experimental technique than that a number of quantitative differences between alexin and opsonin should have given so very similar results.

On looking for a common technical cause of the systematic divergence between the mutual relation of the curves of dilution and inhibition experiments it is most natural to examine the points of difference between the bactericidal and phagocytosis experiments. The most important of these points was mentioned already in the course of the discussion of the distribution of leukocytes and its influence on the shape of phagocytosis curves (see page 139). It was emphasized there that, unlike the result of a bactericidal experiment, the result of a phagocytosis experiment is not derived from direct observation of the condition of the bacteria, but that the latter is determined indirectly from the reaction between bacteria and leukocytes.

When the result of a bactericidal experiment is read after a given period and a percentage of survival of for instance 50 is found, it is obvious that this result does not depend on the time when the bacteria were killed. It may equally well have been at the beginning and at the end of the experiment; i.e. the result is independent of the lag phase of the alexin process and is brought about if only, within the experimental time, the process

reaches an intensity sufficient to produce the observed 50 per cent.-killing.—In the case of the phagocytosis experiments conditions are not so simple: The degree of phagocytosis is not only a function of the phagocytability acquired by the bacteria under the influence of serum but also of the time available for the establishment of contact between bacteria and leukocytes. It is, therefore, evident that the degree of phagocytosis will depend on when exactly in the course of the experiment the bacteria are exposed to the influence of opsonin. If it happens at the beginning of the experiment, marked phagocytosis may be expected as a result of the favourable combination of great phagocytability *and* a long time for phagocytosis. If, on the other hand, there is a lag phase before the opsonin activity makes itself felt, the degree of phagocytosis will be less, because the time at disposal *after* the bacteria have acquired great phagocytability is shorter than before.—A factor which may conveniently be termed “the effective time for phagocytosis” thus depends on how soon in the experiment the opsonin will influence the bacteria, thereby producing increased phagocytability.

At first it seems to be a necessary consequence of the identity theory that the same degree of phagocytosis would always correspond to a given degree of bactericide. The above considerations show that this is not so. If for instance two experiments—one with diluted serum and one with serum + citrate—give the same percentage of survival after 30 minutes, it will be realized *that the corresponding degrees of phagocytosis can only be expected to be equal, if the opsonin activity follows the same time curve in the two experiments.*

It was explained in Chapter III how an evaluation of the alexin- or the alexin + opsonin activity may justly be based upon a single reading taken at a suitable moment. If, however, a comparison between the serum activity in phagocytosis experiments of different composition is wanted (as is the case in the titration experiments in Chapter X) it will be necessary, as shown by the above conclusion, to know the course of the serum activity within the experimental time in the tubes compared. It follows from the identity theory that alterations of the intensity of the alexin and opsonin activity in the course of the experiments must

keep abreast; therefore, on a curve showing the relation between the intensity of alexin activity and time, it should be possible also to read possible fluctuations of the opsonin activity. In order possibly to arrive at a more complete understanding of the titration experiments an investigation into the course of the alexin process within the experimental time was carried out. This course was determined partly in milieu containing serum of varying dilution and partly in milieu containing serum + citrate, serum + anticomplementary serum, or with partially reactivated NH_3 -serum.

The investigations into the relation between time and alexin activity were carried out with the following technique: The mixtures whose activities have to be compared are prepared in bottles (e.g. one with 2.0 cc. active serum + 0.2 cc. 10 per cent. citrate + 1.8 cc. saline solution, and for comparison a bottle containing 0.5 cc. active serum + 1.5 cc. heated serum + 2.0 cc. saline solution). The bottles are placed in incubator at 37° and at intervals of 1 minute 1.0 cc. bacterial suspension is added to each bottle; the fluids are mixed thoroughly, care being taken to avoid the formation of too much foam. From the moment when bacteria are added and at fixed intervals, portions of 0.5 cc. are removed from the bottles with clean pipettes and transferred to test tubes containing 8.0 cc. cold saline solution. Centrifugation after the interruption of the reaction is unnecessary. The initial value of the experiment is determined from a control test tube containing 8.4 cc. saline solution + 0.1 cc. bacterial suspension. In the following experiments the bactericidal degree was determined after 5, 10, 15, 20, 30, 40 or 45 and 60 minutes.

As will be seen from the above example, experiments with serum + inhibitory agent were combined with control experiments with diluted serum. The different experiments showing the course of the alexin process in diluted serum thus have to be collected from several experiments of different days; all the experiments agree well and are reproduced together in Table 47. —Special considerations led to the choice of the manner of graphic representation: It has been shown earlier that a bactericidal titration, reproduced in a probit diagram with the logarithm of the concentration of fresh serum as abscissa, usually

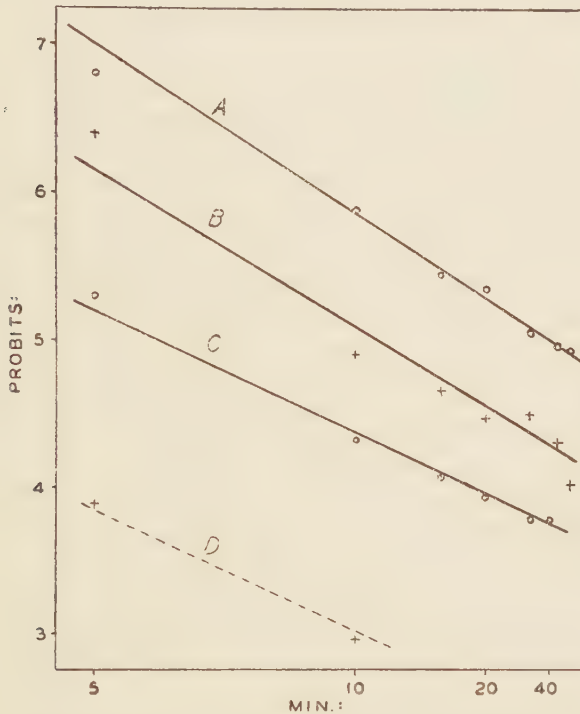
gives a straight line. This means that, if for instance the concentration of serum is doubled, the same numerical decline of the probit value is produced at any point in the course of the titration. Still, the following determinations of the time course of the alexin process are simply bactericidal titrations with time instead of serum concentration as the variable element; it therefore seems natural to try different simple ways of marking out the time in order to see if the time curve could be reduced to linear shape by simple means. It has been found that if the reciprocal value of the time is used as abscissa, all the time titrations in milieu with diluted serum will be reduced approximately to linear shape. Put into text this means that the killing of bacteria is most marked at the beginning of the experiment and that it diminishes in such a way that the rate of killing at a given moment, which again represents the intensity of the alexin process, is inversely proportional to the time passed since the beginning of the experiment. As will appear from the curves in Table 47, this law applies quite well in the interval from 5 to 60 min., reckoned from the beginning of the experiment, which means that the lag phase of the alexin process in diluted serum is very short. That the process not only has a short lag phase but may also come to an end very soon under suitable conditions is seen from the dotted curve (D), showing that nearly a 100 per cent.-killing of the bacteria may be attained within 10 minutes.

For the purpose of comparison with the experiments with diluted serum described the following experiments with serum + inhibitory agent were carried out: Table 48 (serum + citrate), Table 49 (serum + anticomplementary serum), and Table 50 (partially reactivated NH_3 -serum). Even if the curves found in these three cases are not of exactly the same shape, it is still possible to examine them collectively, as the course of the alexin process in all the inhibition experiments differs from the course in the dilution experiments in the same manner. It is characteristic of all the reactions in inhibited milieu that to begin with the intensity is low as compared with the concentration of active serum and that it diminishes far more slowly than in diluted serum.—In order to facilitate the comparison between the fundamentally different courses of

alexin activity in inhibited and diluted milieus some of the rectilinear curves of Table 47 have been transferred to Tables 48-50 as dotted lines. In this manner it is seen distinctly how a given percentage of survival may result either from an intense initial alexin effect, which soon subsides (dilution experiments),

TABLE 47

Determinations of the Course of the Bactericidal Process within the first Hour in Milieus with Different Activity.



The total conc. of serum the same in all four experiments.

A: Experimental culture: Breslau 206.

B: " " : " 206.

C: " " : " 206A, (the C-curve is based on values from three curves taking a nearly parallel course close to each other).

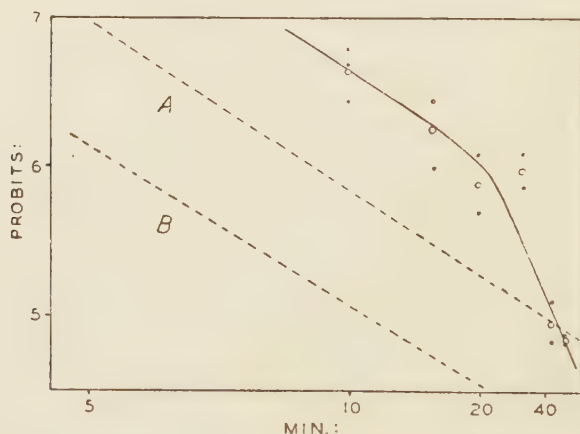
D: Experimental culture: Breslau 206A, (the short D-curve is an example showing how quickly the killing of bacteria may occur when a sensitive strain is exposed to alexin in high conc.).

or from an initially weak but persisting, possibly increasing effect (inhibition experiments). The observed difference between the courses of alexin activity in diluted and inhibited milieus is probably due to the fact that inhibition of the alexin process means that the concentration of a single component is reduced, which apparently first and foremost causes the process to develop more slowly.

In connection with the investigations into the time relations a rather important difference between alexin and complement experiments—i.e. bactericidal and hemolysis experiments—should be pointed out. In the case of hemolysis it will usually be seen that the process continues either until all complement has been expended or all erythrocytes have been hemolysed. In a bactericidal experiment, on the other hand, it is often seen that the killing of bacteria ceases, although considerable amounts of alexin are left in the system. The failure of the remaining alexin

TABLE 48

Determination of the Course of the Bactericidal Process within the first Hour in Milieu with Serum + Citrate.



Mean values marked with o; single determinations with dots.

Reaction milieu: 40 % fresh serum + 0.4 % citrate + saline; total volume 5.0 cc.

Two of the curves of Table 47 represented by dotted lines.

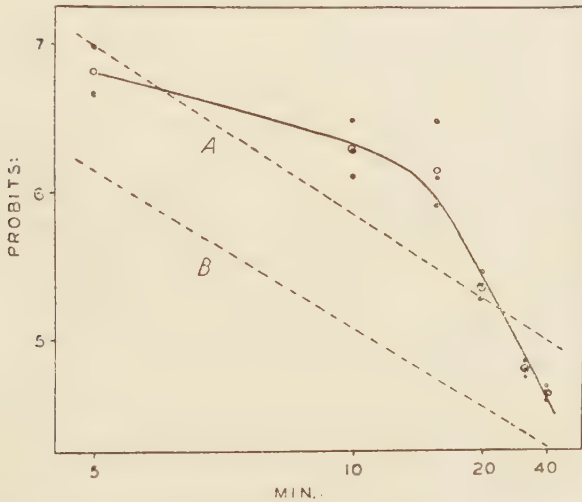
Experimental culture: Breslau 206.

to continue the process in experiments with few bacteria can hardly be attributed to any considerable decline of the alexin concentration; when the process ceases it will sooner be due to the fact that the surviving bacteria possess so great a vitality that the decomposing effect of alexin on the bacterial substance is compensated by the regenerative power of the living bacteria. From this point of view it is also possible to get an idea of why the lag phase and generation period in a suspension of bacteria are extended when alexin is added.

The theoretical considerations on pages 157-158 showed that the moment within the experimental time at which the opsonin activity begins and the rate at which it develops will be among the factors determining the degree of phagocytosis obtained. At

TABLE 49

Determination of the Course of the Bactericidal Process within the first 40 Min. in Milieu with Serum + Anticomplementary Serum.



Mean values marked with o; single determinations with dots.

Reaction milieu: 35 % fresh serum + 0.6 % anticomplementary serum + saline; total volume 5.0 cc.

Two of the curves of Table 47 represented by dotted lines.

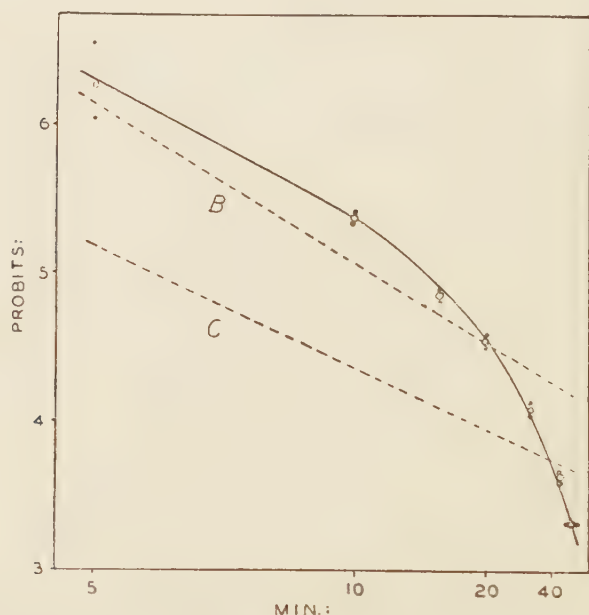
Experimental culture: Breslau 206A.

the same time it was pointed out that, according to the identity theory, fluctuations of the intensity of alexin and opsonin activity must be supposed to take a parallel course in the same milieu. If now it is taken for granted that the time curves in Tables 47 to 50 represent fluctuations of the alexin activity as well as the opsonin activity in the course of the respective experiments, it is possible to test the identity theory by re-examining the titration experiments referred to in Chapter X and see how they agree with the results of the time titrations.

It will be seen immediately that the difference established

TABLE 50

Determination of the Course of the Bactericidal Process within the first Hour in Milieu with partially reactivated NH_2 -Serum.



Mean values marked with o; single determinations with dots.

Reaction milieu: 50 % NH_2 -serum (i.e. 33 % undiluted serum) + 1.6 % heated serum + saline; total volume 5.0 cc.

Two of the curves of Table 47 represented by dotted lines.

Experimental culture: Breslau 206.

between the mutual relations of the curves of dilution and inhibition experiments in Chapter X has found a parallel in the time titrations, where a principal distinction between dilution and inhibition experiments has also been established. The importance of the time titrations is clearly understood from what has been said about the different ways which may lead to the *same* degree of bactericide in a test tube with diluted serum and for instance one with serum + anticomplementary serum. On comparison of these test tubes with corresponding phagocytosis test tubes it is seen clearly from the curves in Table 49 that the "effective time for phagocytosis" is shortest in the inhibited milieu, where the serum activity develops at the slow rate characteristic of all inhibited reactions. This fact involves that the phagocytosis process will make itself less felt in inhibited than in diluted milieu, which again means that the *difference* between the degrees of bactericide and phagocytosis must be greater in diluted than in inhibited milieu, even if, as supposed, the degrees of bactericide are equally great (see for instance Table 42).

CONCLUSION

In contradistinction to the primary impression, gathered from the titration-experiments in Chapter X, it can now be stated:

That the relations between bactericidal and phagocytosis curves in the inhibition titrations, where the curves were always found to lie closer to one another than in the corresponding dilution titrations, do not disagree with the identity theory; on the contrary, it can now be seen that, considering the time factor, the findings in Chapter X are in direct accordance with the identity theory.

It seems necessary to stop at this qualitative conclusion and to be content with demonstrating that when dilution and inhibition experiments are compared, the phagocytosis curve of the inhibition experiment must be expected to display *some* displacement towards the bactericidal curve. If the quantitative influence of the time course of the reactions were to be determined, experiments would be required, not only comprising the usual

dilution and inhibition titrations but also necessarily the substitution of every single determination with a time titration. Such ideal "dynamic" experiments would, however, rapidly outgrow our technical possibilities.

The final conclusion of the present work may be expressed as follows: *After thorough qualitative and quantitative comparisons between alexin and opsonin nothing has been found that does not agree with the identity theory.*

Especially as regards the importance of the fourth component to the alexin and opsonin activity respectively, it has been established that the theory of Gordon & collaborators to the effect that the opsonin activity is independent of the fourth component is not tenable.

According to the above conclusion it seems to be unnecessary to go on using the two terms alexin and opsonin; a single term for "the normal, thermolabile, antibacterial factor in serum" ought to be found instead. The old term complement might perhaps be agreed upon. The word complement is, however, intimately connected with hemolysis and points in the direction of a mechanism of reaction which is no more considered correct; it would therefore be preferable to find and agree upon a new common term for complement, alexin and opsonin.

SUMMARY

PART ONE

CHAPTER I

First the technique employed in phagocytosis determinations in the present work is briefly reviewed. The principal new feature of this technique is that the phagocytosis is determined by means of counting of colonies and not, as in previous investigations, through microscopic observations. The difference between the number of bacteria added to the test tube and the number of supernatant bacteria left in the fluid when the blood corpuscles plus the phagocytized bacteria have been centrifuged down at the end of the experiment is used to denote the phagocytosis degree. It is pointed out that the new technique has certain advantages, especially in attempts at comparing the alexin and opsonin processes, as the effects of both substances is expressed in the same manner, i.e. by means of the disappearance of the living bacteria from the fluid phase. The aim of the work is then expressed as follows:

Examination of the relationship between the normal, thermolabile, antibacterial serum processes.

To avoid misapprehension owing to varying usage it is decided to use the word *alexin* to denote the thermolabile bactericidal normal substance of serum, and the word *complement* to denote the hemolysis-producing normal substance; the term *opsonin* requires no further qualification. The development of the alexin and opsonin doctrine is then briefly reviewed, references being made to the parts of the work where the different problems are dealt with and the more special literature is gone through. On page 20 a survey of the constitution of alexin, complement, and opsonin is outlined.

In conclusion, the risk of performing a work with fundamental questions with a single bacterial strain and a single serum type is discussed.

CHAPTER II

This chapter falls in two parts: First, the course of the ordinary bactericidal and phagocytosis experiments is described and in direct continuation hereof a thorough description is given of how practical exponents of the bactericidal and phagocytosis degrees are computed from the colony figures arrived at—the experimental *values*. On page 32 the methods of computation are briefly summarized.

The second part of this chapter contains detailed information about the single parts of the experiments. In this connection it is pointed out especially how important it is to carry out the washing of the blood corpuscles with a fixed technique, the true concentration of serum in the test tubes thus being known. Besides, the importance of interrupting the experiments effectively and precisely is pointed out. In conclusion the dependence of the phagocytosis process on the temperature is examined, and it is mentioned how the lag phase and tolerance to saline solution of the bacteria employed are of decisive importance to the possibilities of experiments.

CHAPTER III

Like the preceding chapter this one falls in two parts. First, three fundamental questions are discussed, a) the justification of estimating the experiments on the basis of a single reading, b) the possible variation of the number of bacteria within the period of inoculation, c) the possibility that the erythrocytes may take bacteria down with them in the course of centrifugation.

Then a brief reference is made to the ordinary causes of variability and the results of the analysis of the figures; formulae for the computation of "t"- and "P"-values are to be found on page 45.

In the second part of Chapter III the different causes of variability are referred to in detail. It is described how especially the bacterial cultures employed may vary considerably as to sensitivity to serum influence, while the capacity for phagocytosis of the leukocytes and the serum activity are shown to be fairly constant in the same healthy donor. Then the relation between number of bacteria on the one hand and the number of leukocytes or the serum concentration on the other hand is referred to; it is shown that the bacterial suspensions employed are so weak as to allow a considerable increase of the number of bacteria without exposing the leukocytes to "saturation" with bacteria, or causing the serum activity to decrease to any great extent for that reason in the course of the experiment. It is emphasized, as far as all variable factors are concerned, that direct comparisons between the results of different test tubes are permissible only within the same experiment.

Lastly, Chapter III reviews the statistical dealing with the material of figures. It is shown that the homogeneity of the bacterial suspensions at the time of inoculation is independent of the differences in the composition of the experimental milieus that occur. Moreover the withdrawal variation is examined and it is shown to have a variance of very nearly $1.1 \times$ the colony figure; in this connection a few experiments are referred to which, owing to technical flaws, cannot be compared with the remaining material. On pages 64-65 the meaning of the different terms of the expression of the variance arrived at is shown by means of an example.

PART TWO

CHAPTER IV

It is shown that, in contradistinction to previous suppositions, inactive serum is no indifferent medium for bactericidal and phagocytosis experiments, as sufficiently high concentrations of inactive serum will reduce both the bactericidal effect and the spontaneous phagocytosis. It is moreover shown that the inhibi-

tion of phagocytosis is not due to increased viscosity of the experimental milieu but that both the inhibitory effects are presumably brought about by *normal* thermostable substances of the serum influencing the bacteria. It appears from the experiments that the inhibitions occur at a certain concentration of thermostable serum substances, rather independently of the bacterial density as long as the latter is fairly low.

CHAPTER V

It is supposed in this chapter that the inhibitory normal substances referred to in Chapter IV are identical with the so-called "normal antibodies", and the reason is stated why absorption experiments cannot be used to examine the question in detail. Instead of such experiments it is suggested to compare the effects of the normal substances and the immune bodies on the bactericidal and phagocytosis processes.

First, it is shown that experiments with addition of immune body are technically possible (in the weak bacterial suspensions employed no agglutination will occur within the experimental time). Then the well-known Neisser-Wechsberg's zone phenomenon, produced by immune body in high concentrations, is examined.—It is shown that the bactericidal process is inhibited by immune body exceeding a certain concentration and that the inhibition comprises discontinuation both of the stimulating effect of the immune body and of the basal effect that is due to the active serum. Lastly, the effect of the immune bodies on phagocytosis is examined and it is shown that, when the concentration of active serum is not too high, a suitably high concentration of immune body will discontinue all phagocytosis. The conclusion is drawn from the examinations referred to in Chapters IV and V *that probably the inhibitory normal substances are identical with the "normal antibodies"*.

In connection with the experiments with immune serum it is pointed out that Widal's reaction is an insufficient guarantee that the donor's blood does not contain immune body in amounts that may influence a bactericidal or phagocytosis experiment. A safer test is suggested.

PART THREE

CHAPTER VI

The methods hitherto employed for the determination of complement, alexin, and opsonin activities are briefly reviewed, and the advantages of the new technique are pointed out, especially as far as comparisons between alexin and opsonin are concerned. Then the lines which the comparative investigations should follow are stated. These examinations fall in two groups: Qualitative investigations (Chapters VII to IX) and quantitative investigations (Chapters X and XI).

CHAPTER VII

First, Gordon's discovery of the fourth component of complement is mentioned, then follows a discussion of some experiments from which Gordon and collaborators concluded that the fourth component is not required for the opsonin process. It is shown that Gordon's conclusion can hardly be considered sufficiently well-founded.

Before the writer's investigations are referred to, an explanation is given of the principle of the experiments with preliminary treatment that are used in all the subsequent qualitative comparisons between the alexin and the opsonin processes. It is emphasized that with the technique stated it is possible to examine the phagocytability of the bacteria submitted to preliminary treatment immediately after that treatment has been stopped, as it is interrupted by means of great dilution and not, as by previous investigators, by means of centrifugation and washing of the bacteria treated.

It is then shown by means of experiments with preliminary treatment *that the fourth component is required both for the alexin and the opsonin processes*; and it is shown in another experiment that when the preliminary treatment is interrupted all opsonin and alexin effect will cease—there is no after-effect, as can be seen in hemolysis experiments.

In conclusion ether-treatment of serum and its effect on the alexin and opsonin processes are referred to.

CHAPTER VIII

After a short survey of previous experiments with anticomplementary serum a hemolysis experiment is reported, showing that the effect of anticomplementary serum is probably due to the fact that one or both thermolabile components of alexin or opsonin are absorbed by abnormal proteins in the anticomplementary serum.

It is shown by means of an ordinary experiment with preliminary treatment that both the alexin and the opsonin processes are blocked up by anticomplementary serum.

CHAPTER IX

It is emphasized in the reference to previous investigations that nothing is known with certainty about the mode of action of citrate and oxalate, which it is therefore tried to elucidate first: It is shown that addition of citrate will only bring about insignificant changes of the pH and the osmotic pressure and that, probably, the inhibitory effect must be due to a specific influence on the antibacterial serum substances.—In examinations of how citrate, oxalate, and tartrate act on the antibacterial serum activity and coagulation respectively it is shown that, although its Ca-salt is heavily soluble, tartrate does not inhibit any of the two processes. The close correspondence between the antibacterial processes and coagulation is appropriated to prove the perception that the inhibitory effect of citrate and oxalate is due to precipitation of Ca in both cases. It is then discussed how it can be that a number of previous investigators found that citrate and oxalate do not influence the serum activity. The probable reason is shown to be that the said investigators interpose a dilution between the addition of citrate or oxalate and the determination of the serum activity.

Lastly, by means of the ordinary technique of preliminary treatment, it is shown that, in a milieu concentration of about 1 per cent., citrate exercises a strongly inhibitory effect on both the alexin and the opsonin activities.

On page 131 the results of the different qualitative investigations are summarized, which leads to re-adoption of the old hypothesis that alexin and opsonin are identical (*the identity hypothesis*).

CHAPTER X

It is first pointed out that what is observed in the experiments with preliminary treatment doubtless are high degrees of inhibition and not absolute destruction or blockading of the alexin and opsonin processes. Then follows an examination of the theoretical basis of a broader investigation of the *course* followed by the inhibitions, and it is shown that from the identity hypothesis bactericidal and phagocytosis titrations must be expected to result in curves, *the mutual relations of which* are independent of the titration being made by dilution of the serum, or graduation of an inhibitory effect. Two citrate titrations go to show how bactericidal and phagocytosis titration curves as a rule keep fairly closely abreast.

It is then stated that while a bactericidal titration will express the distribution of the bacteria according to alexin sensitivity, a phagocytosis titration cannot be expected direct to express the distribution according to opsonin sensitivity. On the basis of theoretical deliberations it is shown that, as a rule, the interdependence between leukocyte figure and phagocytosis degree and the distribution of the leukocytes according to phagocytic power must influence the shape of a phagocytosis curve.

In conclusion, reference is made to each of the quantitative investigations of the citrate effect, the effect of anticomplementary serum, and of reactivation of NH_3 -treated serum. It is shown that in all the three cases bactericidal and phagocytosis curves are obtained which are situated nearer each other in certain parts than might be expected from the dilution experiments and the identity hypothesis.

CHAPTER XI

It is emphasized that, although the course of the titration experiments does not entirely correspond to what might be

expected from the identity theory, it is striking that *all* the experiments deviate in the *same* manner from what it was expected to find. It is tried to explain the common deviation from conditions relating to the experimental technique: It is shown that, theoretically, the phagocytosis degree must be expected not only to depend on the serum activity but also on how soon the serum influence begins in the experiment. The consequence hereof will be that, even if the identity theory is maintained, it must be expected that different phagocytosis degrees may correspond to a given bactericidal degree, dependent on the course of the serum activity.

Then the course of the alexin process is examined in diluted and inhibited milieus, and it is shown that in this respect, too, there is a fundamental difference between dilution and inhibition experiments. While the alexin activity in diluted milieu is found to be inversely proportional to the experimental time it appears that the process follows a more protracted course in inhibited milieu. This last observation means that the results of the titration experiments in Chapter X do in fact agree with the identity hypothesis, as the protracted course of the serum activity in inhibited milieu will just cause the phagocytosis process to make itself less felt here than in diluted milieu. This again means that the difference between corresponding bactericidal and phagocytosis values must be less in an inhibition experiment than in a dilution experiment, even if the bactericidal values are identical.

In conclusion, all the comparative examinations in Part III are summarized, and it is concluded that all the experiments can be explained in quite a natural manner on the basis of the identity hypothesis.

RÉSUMÉ

AFSNIT I

KAPITEL I

I korte træk gennemgaas først den teknik, der i dette arbejde er benyttet til fagocytosebestemmelserne. Det principielt nye ved den anvendte teknik er, at man vurderer fagocytosen ikke som tidligere ved mikroskopiske iagttagelser men ved hjælp af kolonitælling. Som udtryk for fagocytosegraden anvendes differensen mellem det antal bakterier, der sættes til forsøgsglasset, og det antal, der er tilbage i den ovenstaaende vædske, naar blodlegemerne plus de fagocyterede bakterier ved forsøgets afslutning er centrifugeret ned. Det fremhæves, at den nye teknik byder visse fordele, særlig ved forsøg paa at sammenligne alexin- og opsoninprocessen, idet man faar begge stoffers virkning udtrykt paa samme maade — i.e. ved hjælp af de levende bakteriers forsvinden fra vædskefasen. Arbejdets formaal formuleres derefter saaledes: *Undersøgelse af de normale, termolabile, antibakterielle serumprocessers slægtskab.*

For at undgaa misforstaaelser stammende fra forskelligt sprogbrug vedtages det, at benytte ordet *alexin* som betegnelse for det termolabile, baktericide normalstof i serum, og ordet *komplement* som betegnelse for det hæmolysefremkaldende normalstof; begrebet *opsonin* kræver ingen nærmere afgrænsning. — Herefter gennemgaas kort alexin- og opsoninlærens udvikling, idet der henvises til de steder i arbejdet, hvor de enkelte problemer tages op, og den mere specielle litteratur omtales. Paa side 20 gives i skematisk form en oversigt over alexinets, komplementets og opsoninets sammensætning.

Til slut diskuteres den risiko, der er, ved at udføre et arbejde over principielle spørgsmaal med en enkelt bakteriestamme og en enkelt serumtype.

KAPITEL II

Kapitlet er delt i to afsnit:

Først gennemgaas gangen i de almindelige baktericidi- og fagocytoseforsøg, og i direkte fortsættelse heraf gives en indgaaende beskrivelse af, hvorledes man udfra de fundne kolonital — forsøgsværdierne — beregner praktiske udtryk for baktericidi- og fagocytosegraderne. Paa side 32 er beregningsmetoderne kort resumeret.

I kapitlets andet afsnit gives detaillerede oplysninger om de enkelte led i forsøgene. Herunder fremhæves særlig betydningen af, at blodlegemevaskningen foretages med en fastlagt teknik, saaledes at man kender den sande conc. af serum i forsøgsglassene. Ligeledes paapeges betydningen af, at forsøgene afbrydes effektivt og præcist. Til sidst undersøges fagocytoseprocessens afhængighed af temperaturen, og det omtales, hvorledes de anvendte bakteriers latenstid og tolerance overfor saltvand er af afgørende betydning for forsøgsmulighederne.

KAPITEL III

Kapitlet er ligesom det forrige delt i to afsnit:

Først diskuteres tre principielle spørgsmål, a) berettigelsen i at vurdere forsøgene udfra en enkelt aflæsning, b) bakterietallets eventuelle variation indenfor udsædsperioden, c) muligheden for, at erythrocyterne under centrifugeringen river bakterier med ned.

Derefter omtales kort de almindelige variabilitetsaarsager og resultaterne af talbehandlingen; paa side 45 findes formler til beregning af "t"- og "P"-værdier.

I andet afsnit af kapitel III omtales de enkelte variabilitetsaarsager indgaaende. Det beskrives, hvorledes særlig de anvendte bakteriekulturer kan variere betydeligt m.h.t. følsomhed for serumpaavirkning, mens leucocyternes fagocytoseevne og serumaktiviteten vises at være ret konstante størrelser for samme sunde donor. Derpaa omtales forholdet mellem bakterietal paa den ene side og leucocyttal eller serumconc. paa den anden side; det vises, at de anvendte bakteriesuspensioner er saa tynde, at bakterietallet kan sættes betydeligt op, uden at leucocyterne udsættes for

“mætning” med bakterier, eller serumaktiviteten af den grund falder kendeligt under forsøget. For alle variable faktorerers vedkommende understreges det, at direkte sammenligning mellem resultaterne fra forskellige forsøgsglas kun er tilladelig indenfor samme forsøg.

I kapitel III gennemgaas endelig den statistiske behandling af talmaterialet. Det vises, at homogeniteten af bakteriesuspensionerne paa udsaaaningstidspunktet er uafhængig af de forekommende uensartetheder i forsøgsmilieuernes sammensætning. Videre undersøges udtagevariationen, og det vises, at den har en varians paa meget nær $1,1 \times$ kolonitallet; herunder omtales enkelte forsøg, der p.g.r.a. tekniske brist ikke er sammenlignelige med resten af materialet. Paa side 64—65 vises ved et eks. betydningen af de forskellige led i det fundne udtryk for variansen.

AFSNIT II

KAPITEL IV

Det vises, at inaktivt serum i modsætning til tidligere antagelser ikke er noget indifferent medium for baktericidi- og fagocytoseforsøg, idet tilstrækkelig høje conc. af inaktivt serum ned sætter baade baktericidivirkningen og den spontane fagocytose. Det vises yderligere, at fagocytosehæmningen ikke skyldes forøget viskositet i forsøgsmilieuet, men at begge hæmningsvirkninger antagelig fremkommer ved, at bakterierne paavirkes af *normale*, termostabile stoffer i serum. Det fremgaar af forsøgene, at hæmningerne indtræder ved en bestemt conc. af termostabile serumstoffer ret uafhængigt af bakterietætheden, saalænge denne er nogenlunde lav.

KAPITEL V

Der fremsættes den antagelse, at de i kapitel IV omtalte hæmmende normalstoffer er identiske med de saakaldte “normale antistoffer”, og det begrundes, hvorfor man ikke kan anvende absorptionsforsøg til nærmere undersøgelse af spørgsmaalet. I stedet foreslaas sammenligning mellem normalstofferne og im-

munstofferne virkning paa baktericidi- og fagocytoseprocessen.

Først vises, at forsøg med tilskud af immunstof er teknisk gennemførlige, (i de anvendte tynde bakteriesuspensioner indtræder der ikke agglutination indenfor forsøgstiden). Derefter undersøges med den nye teknik det velkendte Neisser-Wechsberg'ske zonefænomen, der fremkaldes af immunstof i høje conc. — Det vises, at baktericidiprocessen hæmmes af immunstof over en vis conc., og at hæmningen omfatter ophævelse baade af immunstoffets stimulerende virkning og af den basaltvirkning, der skyldes det aktive serum. Endelig undersøges immunstofferne virkning paa fagocytosen, og det vises, at naar conc. af aktivt serum ikke er for høj, vil en passende conc. af immunstof ophæve *al* fagocytose. Af undersøgelserne i kapitel IV og V sluttes, *at de hæmmende normalstoffer sandsynligvis er identiske med de "normale antistoffer"*.

I forbindelse med immunserumforsøgene fremhæves det, at Widal-undersøgelse er en utilstrækkelig garanti for, at donors blod ikke indeholder immunstof i saadanne mængder, at et baktericidi- eller fagocytoseforsøg kan paavirkes deraf. En sikrere test foreslaas.

AFSNIT III

KAPITEL VI

De tidligere anvendte metoder til bestemmelse af komplement-, alexin- og opsoninaktivitet gennemgaas kort, og den nye tekniks fordele, især hvor det drejer sig om sammenligninger mellem alexin og opsonin, fremhæves. Derefter angives retningslinierne for de sammenlignende undersøgelser. Disse falder i to hovedgrupper: Kvalitative undersøgelser (kapitlerne VII—IX) og kvantitative undersøgelser (kapitlerne X og XI).

KAPITEL VII

Først omtales Gordon's opdagelse af komplementets 4' komponent, og derefter diskuteres nogle forsøg, hvoraf Gordon og medarbejdere sluttede, at 4' komponent ikke er nødvendig for

opsoninprocessen. Det vises, at Gordon's konklusion næppe kan anses for tilstrækkelig velunderbygget.

Inden omtalen af egne undersøgelser gennemgaas princippet i de forbehandlingsforsøg, der benyttes til alle de følgende kvalitative sammenligninger mellem alexin- og opsoninprocessen. Det fremhæves, at man med den angivne teknik er i stand til at undersøge de forbehandlede bakteriers fagocyterbarhed umiddelbart efter forbehandlingsens ophør, idet denne afbrydes ved stærk fortynding og ikke som hos tidligere undersøgere ved fracentrifugering og vaskning af de behandlede bakterier.

Ved hjælp af forbehandlingsforsøg vises derefter, at *4' komponent er nødvendig for baade alexin- og opsoninprocessen*; og i et nyt forsøg vises, at naar forbehandlingen afbrydes, ophører al opsonin- og alexinvirkning — der er ikke nogen eftervirkning, saaledes som man kan se i hæmolyseforsøg.

Til slut omtales ætherbehandling af serum og dennes virkning paa alexin- og opsoninprocessen.

KAPITEL VIII

Efter en kort oversigt over tidligere forsøg med antikomplementært serum gennemgaas et hæmolyseforsøg, der viser, at det antikomplementære serums virkning rimeligvis beror paa, at den ene eller begge alexinets eller opsoninets termolabile komponenter beslaglægges af abnorme proteiner i det antikomplementære serum.

Ved hjælp af et almindeligt forbehandlingsforsøg vises, at saavel alexin- som opsoninprocessen blokeres af antikomplementært serum.

KAPITEL IX

Under omtalen af tidligere undersøgelser fremhæves, at man ikke ved noget sikkert om citratets og oxalatets virkemaade, som derfor først søges klarlagt: Det vises, at citrattilskud kun bevirker betydningsløse ændringer af pH og af det osmotiske tryk, og at den hæmmende virkning sandsynligvis maa bero paa en specifik paavirkning af de antibakterielle serumstoffer. — Ved undersøgelser af, hvordan citrat, oxalat og tartrat virker paa henholds-

vis den antibakterielle serumaktivitet og paa koagulationen, vises, at tartrat, skønt dets Ca-salt er tungtopløseligt, ikke hæmmer nogen af de to processer. Den nøje overensstemmelse mellem de antibakterielle processer og koagulationen tages til indtægt for den opfattelse, at citratets og oxalatets hæmmende virkning i begge tilfælde skyldes Ca-fældning. Derefter diskuteres aarsagen til, at en række tidligere undersøgere har fundet, at citrat og oxalat ikke paavirker serumaktiviteten. Det vises, at grunden sandsynligvis er, at de omtalte undersøgere indskyder en fortynding mellem citrat- eller oxalattilsætningen og maalingen af serumaktiviteten.

Endelig vises med den sædvanlige forbehandlingsteknik, at citrat ved en milieuconc. paa ca. 1 % virker stærkt hæmmende baade paa alexin- og opsoninaktiviteten.

Paa side 131 resumeres resultaterne af de forskellige kvalitative undersøgelser, og man føres derigennem tilbage til den gamle hypothese, at alexin og opsonin er identiske (*enhedshypotesen*).

KAPITEL X

Det pointeres først, at det, der iagttages i forbehandlingsforsøgene, sikkert er høje grader af hæmning og ikke absolut destruktion eller blokering af alexin- og opsoninprocessen. Derefter gennemgaas det teoretiske grundlag for en bredere undersøgelse af hæmningsforløbene, og det vises, at man udfra enhedshypotesen maa vente, at baktericidi- og fagocytosetitreringer giver kurver, *hvis indbyrdes relationer* er uafhængige af, om titreringen foretages ved fortynding af serum eller ved graduering af en hæmningsvirkning. — To citrattitreringer benyttes til at vise, hvorledes baktericidi- og fagocytosetitreringskurver som regel følges ret nøje.

Det omtales derefter, at mens en baktericidititrering fører til et udtryk for bakteriernes fordeling efter alexinfølsomhed, kan man ikke vente, at en fagocytosetitrering umiddelbart skal give udtryk for fordelingen efter opsoninfølsomhed. Udfra teoretiske overvejelser vises det, at afhængigheden mellem leucocyttal og fagocytosegrad og leucocyternes fordeling efter fagocytoseevne som regel maa influere paa en fagocytosekurves form.

Endelig gennemgaas hver for sig kvantitative undersøgelser af citratvirkningen, det antikomplementære serums virkning og af reaktiveringen af NH_3 -behandlet serum. Det vises, at i alle tre tilfælde faar man baktericidi- og fagocytosekurver, der i visse afsnit ligger nærmere hinanden, end man skulde vente efter fortyndingsforsøgene og enhedshypotesen.

KAPITEL XI

Det fremhæves, at skønt forløbet af titreringsforsøgene ikke svarer helt til, hvad man efter enhedshypotesen skulde vente, er det paafaldende, at *alle* forsøgene paa *samme* maade afviger fra det forventede. Den fælles afvigelse søges forklaret udfra for-
søgstekniske forhold: Det vises, at man teoretisk maa vente, at fagocytosegraden ikke blot er afhængig af serumaktiviteten men ogsaa af, hvor tidligt i forsøget serumpaavirkningen sætter ind. Følgen heraf bliver, at selvom enhedshypotesen opretholdes, maa man vente, at der til en given baktericidigrad kan svare forskellige fagocytosegrader afhængigt af serumaktivitetens tidsmæssige forløb.

Derefter undersøges experimentelt alexinprocessens tidsmæssige forløb i fortyndet og i hæmmet milieu, og det vises, at der ogsaa i denne henseende er en principiel forskel mellem fortyndings- og hæmningsforsøg. Mens man finder, at alexinaktiviteten i fortyndet milieu er omvendt proportional med tiden, viser det sig, at processen i hæmmet milieu har et mere protraheret forløb. Denne sidste iagttagelse betyder, at resultaterne af titreringsforsøgene i kapitel X er i god overensstemmelse med enhedshypotesen, idet serumaktivitetens protraherede forløb i hæmmet milieu netop vil medføre, at fagocytoseprocessen her gør sig mindre gældende end i fortyndet milieu. Dette betyder igen, at differensen mellem samhørende baktericidi- og fagocytoseværdier maa blive mindre i et hæmningsforsøg end i et fortyndingsforsøg, selvom baktericidiværdierne er ens.

Til slut resumeres alle de sammenlignende undersøgelser i afsnit III, og det konkluderes, at samtlige forsøg paa naturlig maade lader sig forklare udfra enhedshypotesen.

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